Pathophysiological Pro-Inflammatory and Pain-Inducing Mechanisms in Degenerative Intervertebral Discs

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr.sc.nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät
der

Universität Zürich

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Zürich, 2008

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SUMMARY

The intervertebral disc (IVD) is thought to be the source of specific back pain as it exhibits distinct age-related (degenerative) alterations. A compromised nutrition can cause serious cellular and tissue stress altering the phenotype of IVD cells and their embedding extracellular matrix (ECM). These alterations can be mediated partly by oxidative stress which involves post-translational protein modifications at cellular and ECM level in the IVD. Such modifications include the deposition of advanced glycation end-products (AGE) and tyrosine nitration mediated by the highly oxidising agent peroxynitrite. Their presence or accumulation can potentially affect a vast number of cellular processes ranging from pro-inflammatory signalling cascades to a disruption of tissue homeostasis. Nuclear factor κB (NF-κB) is a transcription factor that regulates the expression of hundreds of target genes in response to numerous stimuli. However, aberrant regulation of NF-κB is associated with many inflammatory and degenerative diseases.

The aim of this thesis was to elucidate the physiological impact of oxidative stress in terms of AGE and tyrosine nitration in cells from the nucleus pulposus (NP), centre of the IVD; both of which induced nuclear translocation of the large subunit of NF-κB *i.e*. p65(RelA) in NP cells. The activated targeted genes varied according to the stimuli. AGE induced MMP-13 expression implying a role in matrix remodelling, whereas peroxynitrite as the source of tyrosine nitration induced a pro-inflammatory response involving a prolonged p65(RelA) nuclear shuttling. Taken together, these results imply that NF-κB activation plays a key role and can potentially influence ageing/degeneration processes in the IVD through AGE accumulation or peroxynitrite action.

ZUSAMMENFASSUNG

Die Bandscheibe durchläuft mit zunehmendem Alter degenerative Veränderungen, die an der Entstehung von Rückenschmerzen beteiligt zu sein scheinen. Die kompromittierte Ernährung in der Bandscheibe kann schwerwiegenden Stress in Zellen und Gewebe auslösen, der zu Veränderungen im Phänotyp der Zellen und in der extrazellulären Matrix führen kann. Diese Veränderungen können auf oxidativem Stress basieren und zu posttranslationalen Proteinmodifikationen in Zellen und Matrix führen, z.B. zur Bildung von Advanced Glycation End Products (AGE) oder zur Nitrierung von Tyrosin durch Peroxynitrit. Die Anreicherung dieser Produkte kann u.a. eine proinflammatorische Kaskade aktivieren oder das Gleichgewicht im Gewebeumbau stören. Der nukleare Faktor kB (NF-kB) ist ein Transkriptionsfaktor, der die Expression vieler Gene reguliert und mit einer Vielzahl inflammatorischen und degenerativer Erkrankungen verknüpft ist.

Das Ziel dieser Doktorarbeit war es die physiologischen Auswirkungen von oxidativen Stress (und damit das Vorkommen von AGE und die Nitrierung von Tyrosin) auf die Zellen der inneren Bandscheibenregion (Nukleus Pulposus) zu untersuchen.

Sowohl das Vorkommen von AGE, als auch die Nitrierung von Tyrosin führt in den Zellen des Nukleus Pulposus zur nuklearen Translokation der grossen Untereinheit von Nf-kB (= p65 = RelA). Die aktivierten Zielgene variierten hierbei je nach Stimuli: AGE induzierte die Genexpression von MMP-13 (was auf eine Bedeutung im Matrixumbau hindeutet), während Peroxynitrit eine proinflammatorische Reaktion verursachte, die mit einer anhaltender Verschiebung von p65 in den Zellkern einherging. Dies Ergebnisse zeigen, dass sowohl AGE als auch Peroxynitrit in der altersbedingten Veränderung der Bandscheibe eine Rolle spielen könnte.

ABBREVIATIONS

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AF	Annulus fibrosus
AGE	Advanced glycation end products
BAFF	B-cell activating factor
C-terminal	Carboxy-terminal
CARM	Coactivator-associated arginine methyltransferase
CBP	CREB-binding protein
CILP	Cartilage intermediate layer protein
CML	N-(carboxymethyl)lysine
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EP	Endplate
GAG	Glycosaminoglycan
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IC	Intracellular
IκB	Inhibitory κB
IKK	IκB kinase complex
IL	Interleukin
IVD	Intervertebral disc
LBP	Low back pain
LTβ	Lymphotoxin β
LPS	Lipopolysaccharide
MDa	Megadalton
MMP	Matrix metalloproteinase
MSK	Mitogen- and stress-activated kinase
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-kappaB
NGF	Nerve growth factor
NIK	NF-κB-inducing kinase

NLS	Nuclear localisation sequence
NO	Nitric oxide
NP	Nucleus pulposus
N-terminal	Amino-terminal
PAGE	Polyacrylamide gel electrophoresis
P/CAF	p300/CBP associated factor
PAMPs	pathogen-associated molecular patterns
PARP-1	Poly (ADP-ribose)polymerase-1
PCM	Pericellular matrix
PCR	Polymerase chain reaction
PKA	Protein kinase A
PGE2	Prostaglandin E2
PLA2	Phospholipase A2
PRMT	Protein arginine methyltransferase
P-TEFb	Positive transcription elongation factor b
RNA	Ribonucleic acid
RHD	Rel homology domain
RPS3	Ribosomal protein 3
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SIN-1	3-Morpholinosydnonimine hydrochloride
SRC	Steroid receptor co-activator
TAD	Transcription activation domain
TAF	TBP-associated factor
TBP	TATA-binding protein
TGF	Transforming growth factor
TIMP	Tissue inhibitory metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
UV	Ultraviolet

INTRODUCTION

1. The human intervertebral disc

1.1. The spine

The spine is a multi-segmented column composed of 24 individual vertebrae plus the sacrum, which can be divided into four regions: cervical, thoracic, lumbar and sacral. The main features of the cervical and lumbar spine are their mobility in these regions and their ability to bear substantial loads, whereas the thoracic spine is less mobile as it forms part of the ribcage. The sacrum is composed of five fused vertebrae and the coccygis consists of four fused vertebrae. The sacrum articulates with the pelvis at the sacroiliac joints [1]. The basic functional unit of the spine is referred to as the 'spinal unit' or 'motion segment' which displays the typical mechanical features of the entire spine. This so-called three joint complex, consists of two adjacent vertebrae separated by the intervertebral disc (IVD) anteriorly and the bilateral facet joints posteriorly. The vertebrae are further connected by spinal ligaments, joint capsules and segmental muscles.

The spine provides stability and mobility to the body and ensures protection to nerve roots and the spinal cord, consequently, its function relies fully on the integrity and interplay of its constituents.

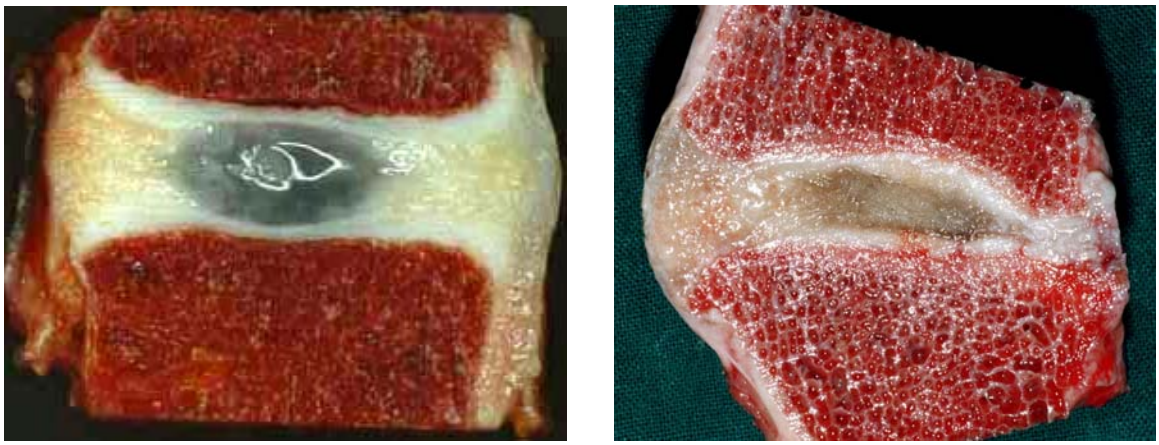
1.2. The healthy intervertebral disc (IVD)

The IVD is a highly specialised tissue that lies between the vertebral bodies. The human IVD consists of three distinct regions: a cartilaginous endplate (EP), the annulus

fibrosus (AF) and the nucleus pulposus (NP) with a transitional zone which merges the latter two regions. Proper biomechanical and biological interaction between the AF and the NP enables the disc to perform various functions; such as bending, flexibility, load distribution, and shock absorption. IVDs represent one third of the height of the spine [2, 3], for instance, the IVD of the lumbar spine has an approximate radius of 20 mm and a thickness of 7-10 mm.

The morphology of disc cells varies across the tissue from a fibroblast-like (ellipsoidal) to chondrocyte-like (round) phenotype in the AF to NP respectively. Adult disc cells represent approximately 1% of the disc volume and are responsible for producing and maintaining the extracellular matrix (ECM) in which they are embedded [4]. The ECM composition also varies across the IVD according to its specific function. There is a predominant gradient of collagen types I and II across the disc. Collagen type I is predominantly encountered in the AF and decreases in concentration towards the transition zone. Collagen type II instead is highly predominant in the NP and decreases progressively in density towards the AF [5].

Matrix maintenance and homeostasis are highly dynamic processes as the ECM is constantly being remodelled leading to a fine tuning between anabolic and catabolic processes. Homeostasis is essential in procuring an adaptational response to changing environments. The interplay that enables homeostasis consists of ECM degradative enzymes such as matrix metalloproteinases (MMPs) and aggrecanases (like ADAMTs) as well as their corresponding inhibitors. In addition, all enzymes and inhibitors seem to be synthesised by disc cells themselves [6].



A B

Figure 1. Intervertebral disc specimens showing the extreme course of ageing in a motion segment of the lumbar spine. A) A sagittal section through the lumbar spine of a 14-year-old infant shows a clear distinction between the nucleus pulposus (NP) and the annulus fibrosus (AF). The cartilaginous endplates (EPs) are composed of a thick layer of hyaline cartilage. The vertebral bodies show rounded edges. B) Conversely, the specimen corresponding to a 70-year-old individual possesses a completely collapsed disc, no distinction between the components of the motion segment is observed. The cartilage enplates are partially resorbed and exhibit severe sclerotic alterations (Courtesy Prof. Boos).

1.2.1. IVD composition and constituents

The annulus fibrosus (AF) is a densely organised structure containing approximately 20 concentric rings. These consist mainly of collagen type I fibril sheets or lamellae which are parallel to the circumferences of the disc, thereby, surrounding the NP. The lamellae are perpendicular in direction to one another and ellipsoidal cells are embedded within this structure. These cells have their major radii aligning with the oriented collagen fibres within the lamellae [7, 8]. The key functions of these lamellae are to retain and restrain the NP. In addition, collagen type I provides tensile properties to the AF and enables it to take up and distribute the load exerted by this tissue during physiologic joint motions. The collagen fibres are cross-linked by a number of molecules bound at their surface which provide

optimal mechanical stability. These stabilising molecules include collagen type X, elastin, fibromodulins, decorin and lumican among others [9, 10].

The nucleus pulposus (NP) instead is a gelatinous structure that occupies the central part of the IVD and is surrounded by the AF. Cells in the NP are initially notochordal but are gradually replaced during childhood by chondrocyte-like cells. Notochordal cells contain large vacuoles and prominent cytoskeletal elements as described by *in situ* studies [11-13]. These are distinguished by their biosynthetic profile and unique biologic responses to mechanical stimuli. It has been suggested that the NP becomes populated at low densities by chondrocyte-like cells that may migrate from the adjacent endplate or inner regions of the AF [14]. NP cells are usually located inside lacunae in the surrounding matrix [15].

A loose and irregular scaffold formed of collagen type II and elastin fibers supports a network of proteoglycans (*e.g.* aggrecan and versican) which provides osmotic properties by retaining water molecules in the disc [9]. This is achieved by multiple, negatively charged glycosaminoglycan side chains (chondroitin and keratan sulphate) that provide resistance to compression and water flow, *i.e.* swelling pressure [16]. Disc hydration represents a key factor in joint loading, muscle activation, and motions of the IVD as it generates high magnitudes of fluid and osmotic pressures, interstitial fluid flows, and compressive, tensile, and shear stresses and strains within the tissue. The ECM of the NP is proteoglycan-rich with aggrecan being one of its main constituents. Aggrecan is comprised of approximately 100 chondroitin sulphate chains, each containing polysaccharide chains with almost 100 negatively charged groups [17, 18]. These polysaccharides are built from some fifty repeat disaccharides that contain one carboxyl and one sulphate negatively charged group. The additional 30 keratan sulphate chains are clustered beside the

chondroitin sulphate chain [19]. The polysaccharide chains are covalently bound to a protein core, which has an N-terminal globular domain that specifically binds hyaluronan, *i.e.* allowing the formation of aggregates [20]. Whereas the C-terminal domain can bind fibulins, tenascins and other matrix components which network to other assemblies in the disc [21]. A high proportion of free aggrecan, *i.e.* not bound to hyaluronan, is present in the NP when compared to articular cartilage [22]. Apart from the abundant collagen type II, other types of collagens are also present in the NP, such as collagen types VI and XI [23]. Collagen type VI is particularly important in the IVD, especially in the NP, as it forms a beaded filament network that plays a role in organising the closest matrix to the cell *i.e.* the pericellular matrix (PCM). It has been postulated that the function of the PCM might be to ensure the biomechanical properties that NP cells have to withstand shear forces [24].

Hyaline, cartilaginous, avascular and aneural endplates separate the IVD, *i.e.* NP and AF, from the adjacent vertebral bodies. The composition of the endplates includes mainly collagen type II, III, V, VI, IX and X, although it may vary with ageing [25]. Integrity of this structure is vital for the diffusion of metabolites in and out of the IVD. Its functions are to partially absorb the hydrostatic pressure dissipated by the NP under loading as well as to prevent it from bulging into the vertebral bodies [26].

1.2.2. IVD physiology

Due to its enclosed nature, the IVD is the largest avascular tissue in the body. Although some capillaries and nerve endings might be present in the most outer layers of the AF, it has been suggested that the main source of metabolites are the capillary beds of the adjacent vertebral bodies which must traverse the endplate to reach the disc matrix [27,

28]. The avascularity of this tissue highlights one of the key features of IVD nutrition and metabolism. There is a gradient of oxygen tension and glucose across the IVD which reaches the lowest levels in the centre of the IVD. Waste products, such as lactic acid, arising from an anaerobic glycolytic metabolism, get diffused in the same manner outwards [29]. This diffusion rate conditions the sparse density of IVD cells across the tissue.

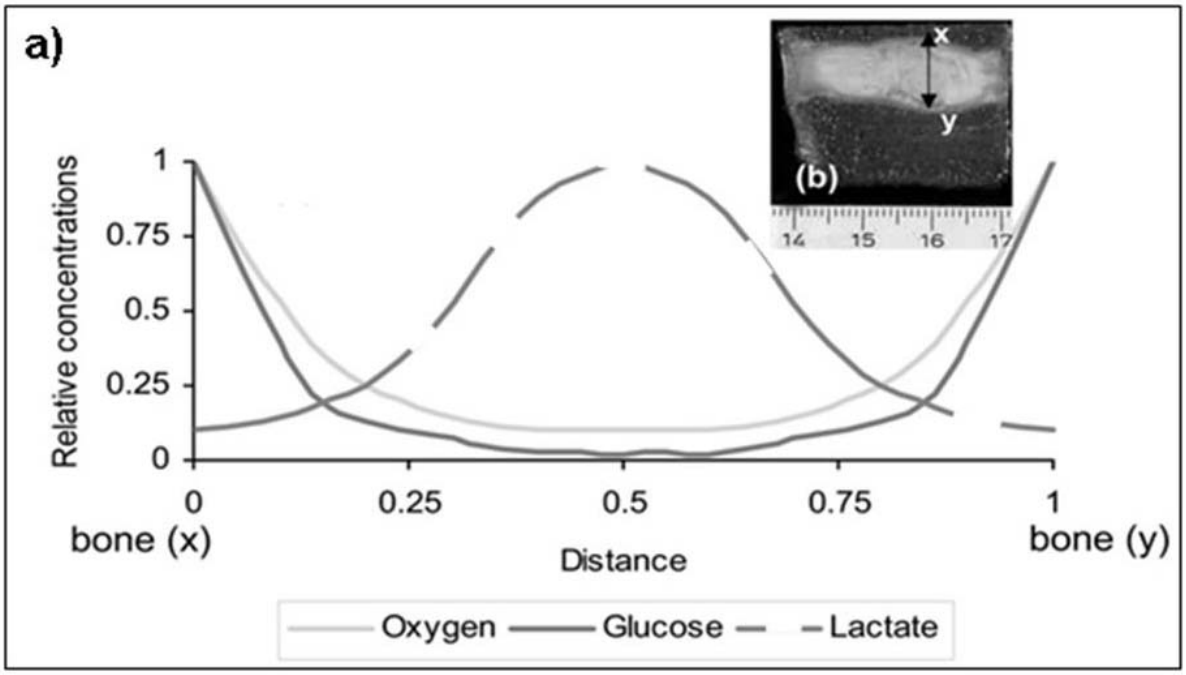


Figure 2. Nutrition in the intervertebral disc. a) Glucose and oxygen concentration decreases gradually from the endplate towards the centre of the nucleus pulposus. Lactate concentration displayed the opposite course, reaching the highest levels in the inner region of the nucleus pulposus. This profile reflects the nutrient and the low pH in the inner region of the disc. b) The sagittal section through the intervertebral disc shows the region of the determined concentration (x to y). Picture taken from [30].

1.3. Age-related (degenerative) changes in the IVD

Disc degeneration is a gradual process graded on the basis of gross morphological and histological changes [31-33], which is inevitable as a result of aging [34]. However, the extent to which such alterations occur in a given individual at a given time are largely variable. First signs of tissue degeneration are already detected in the second decade of life. As the IVD ages, there is a progressive destruction of the disc tissue, starting at the NP and extending to the AF. Both, ECM and cellular changes are observed, finally leading to a loss of the IVD structure and a subsequent replacement by a scar-like tissue [35-39].

We concur with Vernon Roberts that differentiating age-related changes from secondary degeneration is very difficult if not impossible [40]. Within this thesis the term "*age-related changes*" is synonymously used with *disc degeneration* without implying a correlation with pain. In a clinical setting, however, a differentiation of painful and painless disc alterations is warranted. Extensive MRI studies have shown that morphological criteria have failed to allow for such a distinction [41]. Today, painless discs can be differentiated from painful discs by means of provocative discography [42]. However, in this context, *pathological disc*, *i.e.* pain producing discs are differentiated from painless discs. Provocative discography used to diagnose painful disc alterations is still a topic of debate, since the mechanism underlying the reproduced pain is not fully understood [43]. Furthermore, this diagnostic test is not an objective assessment but instead relies on the patients' response which might be influenced by confounding variables such as psycho-social and work-related factors [44].

1.3.1. Aetiology of age-related (degenerative) changes of the IVD

Although the changes and features that occur in the disc during the process of degeneration have been well described (sections 1.3.2., 1.3.3., 1.3.4. and 1.3.5.), however, the aetiology is still not clear. Degeneration of the IVD is thought to be a multi-factorial process involving a mechanical and a genetic predisposition as well as biological factors.

Mechanical factors such as repetitive manual lifting tasks, vibration exposure, and acute overloading have long been implicated in the aetiology of IVD degeneration. *In vivo* and *in vitro* mechanobiologic studies have clearly demonstrated that mechanical factors can influence the biosynthetic activity of disc cells, altering the expression of key extracellular matrix molecules [45-47].

It was recently demonstrated that additional factors, such as a strong, dominant, genetic predisposition as well as biological/nutritional factors, appear to have deeper effects than just biomechanics [29, 48]. Genetic predisposition greatly influences degenerative changes. In fact, epidemiological studies have described strong family predisposition to disc degeneration and herniation [49-53]. Interestingly, studies on identical twins elucidated that 70% of the explained variance in IVD degeneration can be attributed to genetic inheritance rather than to environmental factors [54]. An association of polymorphisms of various sets of genes and IVD degeneration has been suggested; these include vitamin D receptor, collagen I and IX, cartilage intermediate layer protein (CILP), asporin, aggrecan, MMP-3, *i.e.* mostly involved in ECM homeostasis, as well as cytokines such as IL-1β and IL-6 [55-70]. However, most of the genetic influence remains to be explained, as genetic

predisposition to IVD degeneration implies many genes and it is not possible to distinguish between a minority of people with 'vulnerable' backs and a majority with 'normal' backs.

Impairments in the precarious supply of nutrients may lead to cell death or degenerative changes. These not only severely affect and reduce nutrition, but also increment waste products with a further decrease of oxygen tension in the centre of the IVD (Figure 2.). Thus, an accumulation in lactic acid drastically decreases the pH of the ECM and consequently the synthetic activity of disc cells [29]. Cell culture studies have confirmed that disc cells deprived of oxygen have a greatly reduced metabolic rate, and that a prolonged shortage of glucose can kill them [71]. Nevertheless, a recent *in vivo* study suggests that the links between impaired metabolite transport and IVD degeneration are not straight-forward as one cannot clearly distinguish one condition from another [72].

In summary, disc degeneration is a multi-factorial process which is primarily related to an interplay between genetic predisposition and nutrition. Additional factors, such as mechanical factors, can modulate this interaction causing lesions by abnormal loading or mechanical injuries [73, 74].

1.3.2. Phenotypic and cellular alterations

Disc cells actively remodel the ECM, and thereby, are in charge of producing and maintaining the ECM that embed them. Therefore, it is sensible to assume that degenerative alterations might not only be occurring in the ECM but also in the cells responsible for its synthesis and maintenance. For this however, there is only indirect evidence for phenotypic changes of disc cells occurring during IVD degeneration. Research in this area is greatly hampered by a lack of standard exclusive disc cell reference markers. This cell morphology

varies within the IVD itself as discussed in section 1.2.1. Attempts to phenotypically characterise disc cells have taken into account the main constituents of the ECM of a particular region as well as the environment of the IVD. Disc cells from the NP are referred to as 'chondrocyte-like'cells due to their morphological resemblance to articular cartilage chondrocytes. Proposed NP-cell markers include collagen type II, aggrecan, sox-9, HIF-1α, MMP-2 and GLUT-1 [75, 76]. Additionally, further microarray analyses of rat coccygeal intervertebral discs has shown, via real time RT-PCR, that NP cells have higher levels of annexin A3, glypican A3, keratin 19 and pleiotrophin compared to AF cells [77].

As degeneration progresses, aggrecan-mRNA expression has been reported to substantially decrease[78]. Strikingly, the presence of the phagocytic marker CD68 [79-81] has been detected in disc cells from disc tissue with morphological signs of disc degeneration bearing no exogenous infiltration. Thus, this finding suggests a phenotypic switch of NP cells to a phagocyte-like cell type. In addition, the avascular nature of the disc also favours a phenotypic switch rather than the infiltration of phagocytes from outside the IVD. Nevertheless, further analyses are required to examine the degenerative changes in the rest of the proposed markers.

In addition to these phenotypical changes, cell clustering due to reactive proliferation is observed at an advanced age and advanced stage of degeneration (50-70 years) near clefts[33].

1.3.3. Extracellular matrix (ECM) alterations

As the disc degenerates, there is a fragmentation and net loss of proteoglycans and therefore a loss of water from the nucleus, leading to a reduced osmotic pressure and

dehydration in the tissue [78]. This fragmentation affects primarily the proteoglycans aggrecan and versican [82]. A reduction in proteoglycans is thought to decrease disc height. This feature is particularly marked in the NP, which becomes steadily more fibrous and laminated as proteoglycans are slowly replaced by fibrous proteins such as collagens. The overall collagen content in the NP remains fairly constant over the years, however that of the AF decreases with advancing age [83]. Additionally, qualitative changes involving alterations in the distribution and appearance of various collagen types (such as collagen type IV and X) and various isoforms in the distinct regions of the IVD have been reported [84]. Furthermore, changes affecting the collagen fibres by cross-linking greatly impair remodelling or degradation when they become damaged. 'Non-enzymatic glycation' results in cross-linkings which render the tissue excessively stiff and unable to withstand load; *thus* making it vulnerable to injury. A sign of 'non-enzymatic glycation' of proteins is the yellow-brown appearance associated with ageing of tissues which has been reported in the ageing and degenerated IVD [25] (discussed in section 1.3.5.).

Apart from proteoglycan and collagen proteins, there are non-collagenous proteins occurring abundantly in the IVD. Evidence indicates that some of these non-collagenous proteins such as fibronectin undergo age-related changes too [85]. A fundamental characteristic in the ageing/degenerative process of the IVD seems to be an imbalance in the ECM arising from catabolic and anabolic processes. Degradative enzymes (MMPs and ADAMTS [86]) can be produced at levels that overwhelm the production of their inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs). For instance, degenerating IVD are reported to have increased levels of MMP-3 and ADAMTS-4 (A Disintegrin And Metalloproteinase with ThromboSpondin motifs-4) and remarkably low levels of TIMP-1

and 3 [33, 87]. Moreover, enhanced focii of tissue proteolysis have been shown by *in situ* zymography to be associated with cleft formation and disc tissue disruption [25]. All these remodelling enzymes can be synthesised by IVD cells, however expression may be modulated by cytokines, pro-inflammatory mediators and growth factors, such as TNF-α, IL-1, IL-8, TGF-β, nerve growth factor (NGF) and prostaglandins, which seem to be important players in IVD degeneration and potential pain [85, 86, 88-93]. For example, MMP-3 and MMP-13 were reported to be IL-1β dependent. Instead, TGF-β is mostly associated with matrix disarrangement through its anabolic properties, even though the exact mode of action is yet to be elucidated. This indicates that cytokines and growth factors add a modulatory layer to this age-related or degenerative process.

1.3.4. Cellular and ECM modulation by cytokines and growth factors

Cytokines and growth factors provide a regulatory means as IVD cells have been shown to produce and/or respond to such agents (Table 3). In addition, increasing evidence indicates that some cytokines and growth factors are responsible for the alterations of the disc matrix described in sections 1.3.2 and 1.3.3. [86, 90, 94]. Nevertheless, the expression pattern of these factors is unknown to date. Furthermore there is no clear distinction whether these factors arise as part of the normal age-related degeneration process or due to pathological changes of the disc.

In the IVD, pro-inflammatory cytokines tend to have mostly a catabolic activity (*e.g.* TNF-α and interleukins). Growth factors instead exhibit mostly anabolic effects (*e.g.* TGF-β) [90]. Recent evidence indicates that factors of both classes are induced during age-related degeneration, in particular, TNF-α expression has been found to be enhanced in IVD cells

with increasing age in non-symptomatic degenerated intervertebral discs [95]. Members of the interleukin-1 (IL-1) family were reported to be produced in both non-degenerated and degenerated IVD at basal levels; however expression increased with age-related (degenerative) changes of the IVD. The expression of these cytokines implies important signalling cascades that can be activated such as the induction of MMP synthesis or pro-inflammatory mediators (*e.g*. prostaglandins and neurotrophic factors).

Animal studies using TNF-α support the hypothesis that pro-inflammatory cytokines may initiate both a local inflammatory reaction, by rapid diffusion through nuclear and annular clefts and tears, as well as inflammation in the peridiscal space, which is very well innervated [96] Thus, a link between degenerative processes and the induction of discogenic pain is created.

Besides cytokines, growth factors have also been reported to be synthesised in increasing amounts in degenerated IVD as is the case for TGF-β [85]. TGF-β has potent matrix-inducing activity (anabolic effect) and its expression has been enhanced during degeneration implying a rearrangement of the matrix[85]. This finding indicates a putative role in matrix disarrangement, including the formation of granulation tissue that is characterized by changes in the composition and synthesis of collagen and proteoglycan in the ECM, nevertheless the mechanism of TGF-β induction remains unknown.

Therefore, alterations during degeneration in the expression of cytokines and growth factors may disrupt the delicate homeostasis of the tissue that is essential for the maintenance of a healthy disc matrix leading to ECM alterations.

1.3.5. Oxidative stress

Oxidative stress modulates the formation of non-enzymatic, post-translational glycation *in vivo* [97, 98] providing a means for the cross-linking of proteins. Non-enzymatic glycation begins with a reaction between a reducing sugar and a free amino group on a protein, lipid, or nucleic acid. Initially, chemically reversible Schiff base and Amadori adducts form proportionately to reducing sugar concentration. This initial reaction is an amino-carbonyl type condensation of the reducing sugar with a primary amine. The rate of formation or degradation of the Amadori products depends on the percentage of aldehydic of the sugar; lowest for glucose and highest for ribose and glycoaldehyde [99]. Over time, these Amadori products slowly undergo further rearrangements, dehydrations, and oxidations resulting in the reversible formation of a family of complex structures that are referred to as advanced Maillard reactions including advanced glycation end-products (AGEs). Furthermore, the pH plays a fundamental role in the formation of the distinct types of advanced Maillard reactions [100, 101]. A common modification is generated by oxidative cleavage of Amadori intermediates to form N-(carboxymethyl) lysine (CML) structures. Therefore, age-related accumulation of the post-translational protein modification N-(carboxymethyl) lysine (CML) is regarded as a marker for oxidative stress [102]. Long-lasting proteins such as collagens, elastins and proteoglycans are particularly susceptible to AGE deposition, most likely due to the long half lives of these proteins. Thus, AGE accumulates due to the normal process of aging and is enhanced in hyperglycaemia. Interestingly, age-related changes can be clearly observed in collagen-rich tissues, which tend to loose elasticity and become thickened or sclerosed with age [103]. Collagen fibres undergo substantial modifications as the fibres become (i) highly thermostable, (ii) have

covalently linked fluorophores, (iii) decrease in solubility and (iv) and a reduction in digestibility [104-107]. This means of cross-linking through glycated residues has been strongly related to abnormal cellular function [108]. The contribution of AGEs to age- or hyperglycaemic-related complications might occur through either direct chemical-(covalent cross-link formation) or cell surface receptor mediated-pathways. These complications include disorders such as osteoarthritis, cataract formation, myocardial dysfunction and nephropathy among others [109-112]. A contribution to ageing/degeneration of the IVD would therefore be expected through cross-linking, insolubilisation and free-radical-mediated fragmentation of proteins. CML modifications have been immunolocalised in IVD tissues, revealing an age-dependent CML deposition in areas of macroscopic and histological disc degeneration [25]. CML and other AGE are ligands can be recognised by a number of receptors which include the receptor for advanced glycation end-products (RAGE) [25, 113, 114] and the so-called AGE-receptor complex. To date, RAGE is the best characterised AGE-receptor that has been reported in chondrocytes from articular cartilage to activate NF-κB, presumably through the extracellular-regulated kinase (Erk), Rho, and Jak/Stat pathways [115, 116].

Nevertheless, the origin of oxidants mediating oxidative stress as a prerequisite to induce generation of CML-modification in the low oxygen environment as inside the IVD, remains unclear. Oxidative enzymes might provide the necessary oxidants to induce oxidative stress. A recent study demonstrated that accumulation of CML-modification in matrix proteins is impaired in NADPH oxidase-deficient mice [117], implying that in a low oxygen environment the oxidants generated by NADPH oxidase may be responsible for the generation of CML-modifications [118]. Strikingly, NADPH oxidase is normally produced

by cells of myeloid origin, *i.e.* phagocytic cells, granulocytes, mast cells and dendritic cells [119]. As mentioned above, CD68 positive cells have been identified in the NP of individuals with histomorphological signs of disc degeneration [39] but with no signs of extra-discal infiltration (section 1.3.2.). However, further research is required to find out whether CD68 expressing cells also display other phagocytic features such as expression of active NADPH oxidase.

In addition, AGE deposition has been reported to affect remodelling and destruction of the tissue as well as to confer stiffness and brittleness to a tissue [120, 121].

1.3.6. Consequences of age-related (degenerative) changes

Inevitably, all these changes lead to increased load on the structures surrounding the NP and ultimately the whole spine. Abnormal distribution of forces across the disc results in cracking and fissuring of the AF, further decreasing its ability to distribute stresses properly during loading causing clefts, tears and granular changes to appear in the ECM. The integrity of the EP fails as well with ageing and degeneration. Extensive calcification, ossification and cartilage disorganisation may lead to decreased EP permeability; and thus compromise the IVD greatly [122, 123]. Additional changes may occur which include fissure formation, micro-fractures, horizontal cleft formation and death of chondrocytes. Consequently the disrupted IVD can lead to tissue failure, *e.g.* AF tears, EP damage, or facet-joint damage.

As IVD degeneration proceeds, biomechanical changes in the IVD and the motion segment follow. In the early stages of IVD degeneration, the involved motion segment is less stiff than in the advanced stages, particularly in flexion-extension and torsion [124, 125].

Vertebral body pathology is also a hallmark of late-stage degeneration and includes subchondral sclerosis, end-plate ossification, and osteophyte formation.

1.4. Clinical relevance: low back-pain and IVD degeneration

Low back-pain is common and appears as pain, muscle tension, or stiffness localised below the costal margin and above the inferior gluteal folds, with or without leg pain (sciatica) [126]. Low back-pain is the leading cause of restricted activity and bed rest in the US working population and it is the second leading symptom (after that of common cold) that prompts visits to physicians [127, 128]. Moreover approximately 85% of the population in western industrialised societies experience low back-pain at least once during their lives, underlying its dramatic impact on society [129-131]. Low back-pain has a strong tendency to become chronic, reaching 36% for a 12-month incidence [132]. Chronic low back-pain and its treatment is a growing socio-economic problem in western industrial countries. Waddell has documented a dramatic increase in low back-pain disability in Great Britain that may be representative of Western societies [128].

Low back-pain can be differentiated into *specific* and *non-specific disorders*. Specific back-pain is related to a clear morphological correlate while non-specific back-pain is not. In about 85 to 90% of the individuals, no clear cause of back-pain can be identified despite intensive diagnostic tests. Specific spinal syndromes leading to leg pain are caused by disc herniation (radiculopathy) or a narrow spinal canal (spinal claudication). Among the putative causes of specific back-pain are symptomatic facet joint osteoarthritis, segmental instability and painful disc degeneration. The dramatic changes that occur in the

intervertebral disc with age (Fig. 1) make many clinicians and researchers believe that the disc is a predominant source of back-pain. The so-called *discogenic pain syndrome* is a result of painful disc degeneration. Epidemiological studies using MRI have shown that morphological alterations in the intervertebral disc do not allow differentiation of symptomatic from asymptomatic individuals since almost all morphological alterations can occur in both groups [133]. It is therefore assumed that biochemical alterations are the decisive factor why a degenerated disc may become painful. Such biochemical mechanisms that link morphological alterations in the ECM matrix, especially in the NP, during degeneration with the induction of pain are still unknown. It has been demonstrated that resident disc cells undergo phenotypical alterations (section 1.3.2.) due to the stress imposed on them by the progressively worsening environmental conditions inside the degenerating disc. These alterations lead to altered gene expression patterns of the disc cells, and may induce the expression of mediators that contribute to or cause pain inducing signalling mechanisms. Among potential candidates for such mediators are the pro-inflammatory cytokines. Olmarker and co-workers showed that NP-induced nerve root injury during herniation can be mimicked by a local application of TNF-α to the nerve roots [134-136]. In a degenerated, non-herniated disc it is conceivable that TNF-α produced by NP cells can diffuse out of the disc through clefts and tears. In the periphery, TNF-α might directly or indirectly (via further cytokines) irritate the nerve endings in the outermost part of the AF or the nerve roots in the epidural space. Besides TNF-α, disc cells have been shown to be able to synthesize several additional pro-inflammatory cytokines including IL-1α/β, IL-6, IL-8, and GM-CSF [90, 137]. These findings support the idea that pro-inflammatory cytokines produced by resident disc cells might be involved in the induction

of low back-pain. Despite extensive evidence that pro-inflammatory reactions play a significant role, the mechanisms linking morphological changes during disc degeneration with pain induction responsible for discogenic pain are still not well explored. Furthermore, NF-κB (described in detail in section 2. and 3.) is known to play a central role in the regulation of pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes such as cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) (reviewed in [138]). Therefore, the activation of NF-κB by various stimuli during degeneration might be the starting point for a pro-inflammatory signaling cascade in the intervertebral disc and may be the decisive factor determining whether an intervertebral disc becomes painful and results in the clinical syndrome of discogenic back-pain.

1.5. Experimental models

1.5.1. *In vitro* models

In vitro systems include cell culture (e.g. monolayer and three-dimensional) systems, tissue culture, and organ culture with end plates. Each culture system has its own advantages and disadvantages for specific experiments. Furthermore, the handling and culture of IVD cells has been proved to be complicated. Although it is possible to expand IVD cells in routine monolayer cultures, the cells tend to differentiate and therefore lose their native phenotype [139]. Three-dimensional culture systems of isolated IVD cells helps to circumvent these problems by allowing the maintenance of disc cell morphology, proper cell-cell interactions and providing suitable environmental signals [140]. Several three dimensional-culture systems have been used throughout the literature for IVD cells such as alginate beads, collagen matrices, agarose and fibrin gels, pellets and biocompatible

polymer scaffolds [141-147]. Except for the pellet system which consists of cell aggregates, the other three dimensional-culture systems need to introduce foreign matter to the isolated disc cells, thus alterations of the direct cellular microenvironment might occur; in fact, IVD cells behave differently in each culture system [148]. Considerations in regard to culture supplements such as ascorbic acid and serum may also influence the outcome in an experiment.

Tissue or organ culture might be more applicable in *in vivo* than in cell culture-based models. The main drawback is to keep the cells viable despite changes in the diffusion rate of metabolites, oxygen and substances across the tissue as well as the poor reproducibility of these culture systems.

Besides the *in vitro* model, the source of IVD cells for the study of human pathogenesis of IVD degeneration should also be carefully evaluated. AF and NP cells have diverse biomechanical as well as biosynthetic activities and species specificity, as well as the age and severity of degeneration of the donor IVD and cells can greatly affect the outcome of an experiment.

1.5.2. *In vivo* models

In addition to being able to model human pathologic processes, an animal model must be ethical, controllable, reproducible, and cost-effective. *In vivo* studies are hampered by the invasiveness of the procedures and concerns surrounding the care and use of animals. A variety of *in vivo* animal models for IVD degeneration are described in literature, each of with has its own advantages and disadvantages [149-151]. Small animal models that include rats or rabbits are relatively inexpensive, but the small spinal structure, cell maturity and

cell type may not be ideal for biomechanical or biological experiments. Larger animal models, although more expensive, resemble the mature human IVD from the point of view of the cell biology and biochemistry. The content of notochordal cells and chondrocytes and the transition times from notochordal to chondrocytic cells in the NP vary between species [152]. Generally, the higher the animal species is in the phylogenic tree, the slower the natural process of degeneration.

Naturally occurring IVD degeneration aging models include rats, chondrodystrophoid dogs and primates [153-158], specifically the sand rat has been reported to have very similar degenerative disc changes to humans, occurring spontaneuously and at a young age [159-161].

On the one hand, naturally occurring models have advantages over injury models with regard to the biological changes with ageing and degeneration over time. The main disadvantage of naturally degenerating animal models however is the unknown rate and percentage of degeneration in a group. On the other hand, artificially induced degenerative changes are invaluable in creating clinically applicable and reproducible animal models of degeneration. These injuries tend to lead to rather quick degenerative changes in the IVD when compared to naturally-occurring models. These include postural change, tail suspension, axial loading, knife stab or puncture of the AF-totally or superficially- resection of the lamina and facet joint, EP injury or cementing of discs adjacent to a fusion and chemonucleolysis among others [162-165].

2. Nuclear Factor-KappaB (NF-κB)

NF-κB was initially identified in 1986 as an inducible nuclear factor bound to the immunoglobulin κ light chain enhancer in B-cells [166]. Additional studies however revealed that NF-κB is expressed in almost all cell types [167]. NF-κB is induced by a vast number of very different stimuli ranging from biological substances such as inflammatory cytokines, chemokines, bacterial lipopolysaccharides (LPS) or RNA viruses to phorbol esters and cytotoxic stimuli such as UV light and ionising radiation [168]. Upon induction, NF-κB activates a variety of target genes and thus regulates many diverse biological processes such as the innate and acquired immunity, cell proliferation or apoptosis [168]. Aberrant activation of NF-κB may result in a chronic or exacerbated inflammation associated with many diseases such as rheumatoid arthritis, asthma, atherosclerosis, cancer and Alzheimer's disease [169-173]. This highlights the importance of a tight and coordinated regulation of NF-κB.

2.1. The mammalian NF-κB family members

The NF-κB family of inducible transcription factors in mammals has 5 members, p65(RelA), RelB, c-Rel, p105/p50and p100/p52 encoded by *RELA, RELB, REL, NF-κB1* and *NF-κB2,* respectively [174, 175] (Figure 3).

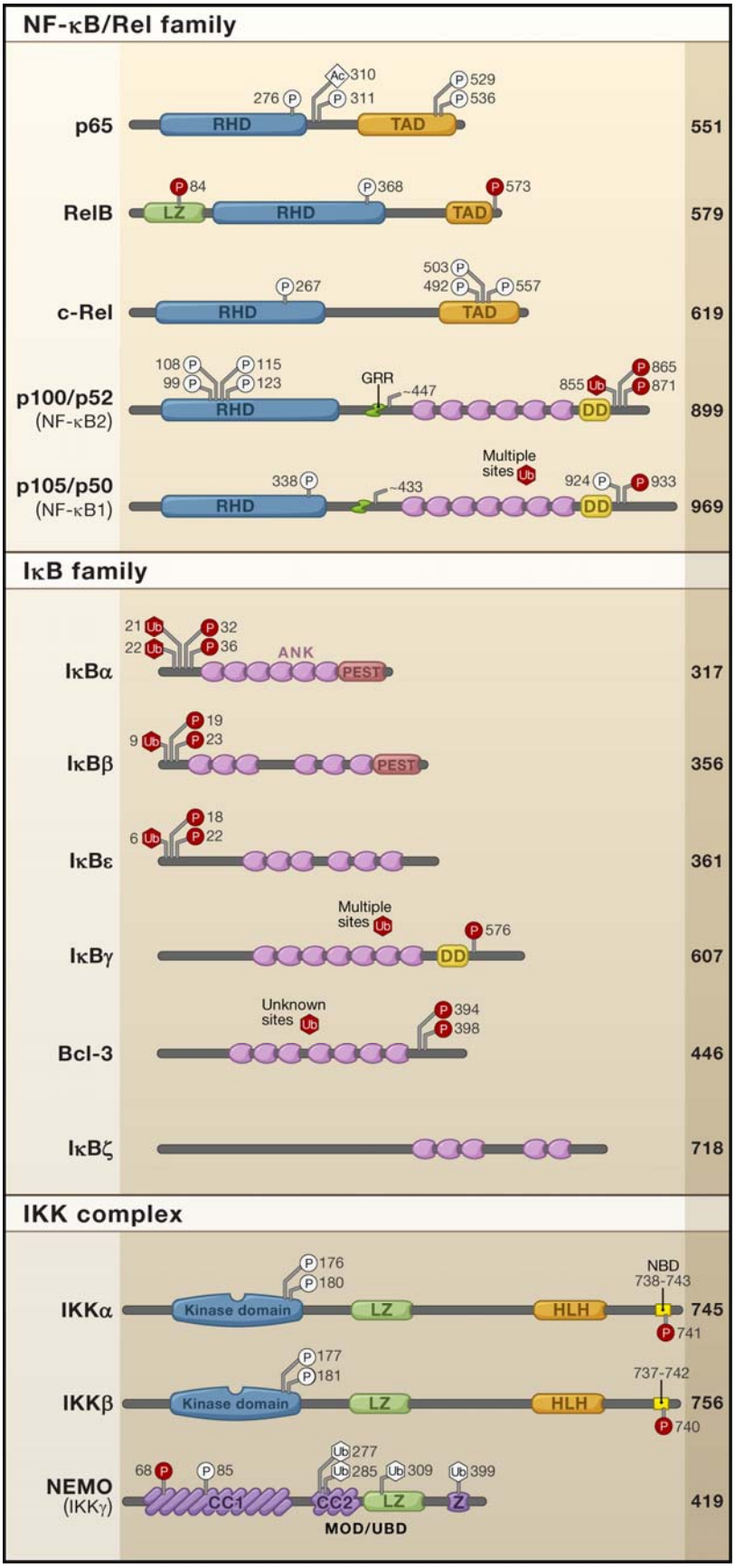

NF-κB/Rel family
p65
RHD
TAD
551
RelB
LZ
RHD
TAD
579
c-Rel
RHD
TAD
619
p100/p52
(NF-κB2)
RHD
GRR
DD
899
p105/p50
(NF-κB1)
RHD
Multiple sites
DD
969
IκB family
IκBα
ANK
PEST
317
IκBβ
PEST
356
IκBε
361
IκBγ
Multiple sites
DD
607
Bcl-3
Unknown sites
446
IκBζ
718
IKK complex
IKKα
Kinase domain
LZ
HLH
NBD
738-743
745
IKKβ
Kinase domain
LZ
HLH
737-742
756
NEMO
(IKKγ)
CC1
CC2
LZ
Z
MOD/UBD
419

Figure 3. The NF-κB, IκB, and IKK protein families. The number of amino acids in each human protein is indicated on the right. Post-translational modifications that influence IKK activityor transcriptional activation are indicated with P for phosphorylation, U for ubiquitination, or Ac for acetylation. Inhibitory events and phosphorylation and ubiquitination sites on p100, p105, and IκB proteins that mediate proteasomal degradation are indicated with red Ps and Us, respectively. RHD, Rel Homology Domain; TAD, transactivation domain; LZ, leucine zipper domain; GRR, glycine rich region; HLH, helix-loop-helix domain; Z, zinc finger domain; CC1/2, coiled-coil domains; NBD, NEMO-binding domain; MOD/UBD, minimal oligomerisation domain and ubiquitin-binding domain; and DD, death domain. Figure taken from [176].

All family members share the Rel Homology Domain (RHD), which is located at the N-terminus and is approximately 300 amino acids long. The RHD mediates DNA binding, dimmerisation, nuclear localisation and interaction with the inhibitory IκB proteins [177]. p65(RelA), RelB and c-Rel are the only family members containing a C-terminal transcription activation domain (TAD), which enables gene activation [178]. In contrast, p50 and p52 contain no TAD but a C-terminal ankyrin repeat domain. p50 and p52 are proteolytically processed from the precursor proteins p105 and p100, respectively [179]. Diverse combinations of NF-κB subunits lead to the formation of homo- and heterodimmers which have different affinities to distinct DNA binding sequences, the κB sites, and regulate differentially specific genes [180]. The best studied heterodimer is p65(RelA)/p50.

2.2. NF-κB signalling pathways

The simplified view of NF-κB regulation involves the retaining of NF-κB in an inactive form in the cytoplasm by IκB proteins; thereby transcriptionally inactivating NF-κB. In response to extracellular stimuli, IκB is phosphorylated by activated IKK, which leads to ubiquitination of IκB by ubiquitin ligases and its degradation by the 26S proteasome, releasing NF-κB dimmers [177]. The MDa IκB kinase (IKK) complex is composed of the kinases IKK1/IKKα, IKK2/IKKβ and the regulatory domain

NEMO/IKKγ (NF-κB essential modulator) which has no intrinsic kinase activity [181-184]. Induction of NF-κB leads to subsequent nuclear translocation of NF-κB dimmers, which will bind to consensus sequences to activate the transcription of target genes. The termination of the response involves a negative feedback due to activated NF-κB dependent IκB expression or ubiquitin-mediated proteosomal degradation of NF-κB [185, 186]. IκB and IKK proteins are central players in the regulation of NF-κB activation. Nevertheless, the established model in which certain IκB family members retain NF-κB dimmers in the cytoplasm, preventing nuclear translocation and subsequent DNA binding is more complex. Despite steady-state localisation that appears almost exclusively cytosolic, there seems to be a constant shuttling between the nucleus and the cytoplasm of the NF-κB/IκB complex [187]. IκB proteins (IκB α, β, ε) contain at the C-terminus protein interaction motifs, referred to as ankyrin repeats, which mediate the interaction with NF-κB [188, 189]. The crystal structure of the NF-κB/IκBα has revealed that IκBα masks only the nuclear localisation sequence (NLS) of p65(RelA), whereas the NLS of p50 remains exposed. The exposed NLS of p50 coupled with nuclear export sequences in IκBα and p65(RelA) leads to this dynamic shuttling between the two compartments.

Additionally, p105 and p100, *i.e.* the unprocessed forms of p50 and p52, have an IκB-like function due to their ability to retain NF-κB dimmers in the cytoplasm via their ankyrin repeats [190]. There are also inducibly expressed IκB family members, Bcl-3 and IκBζ, which function quite differently in the regulation of NF-κB [191, 192].

Upon stimulation, two different types of NF-κB signalling pathways can be activated, the canonical (classical) or the non-canonical (alternative) pathway (Figure 4) [193-195].

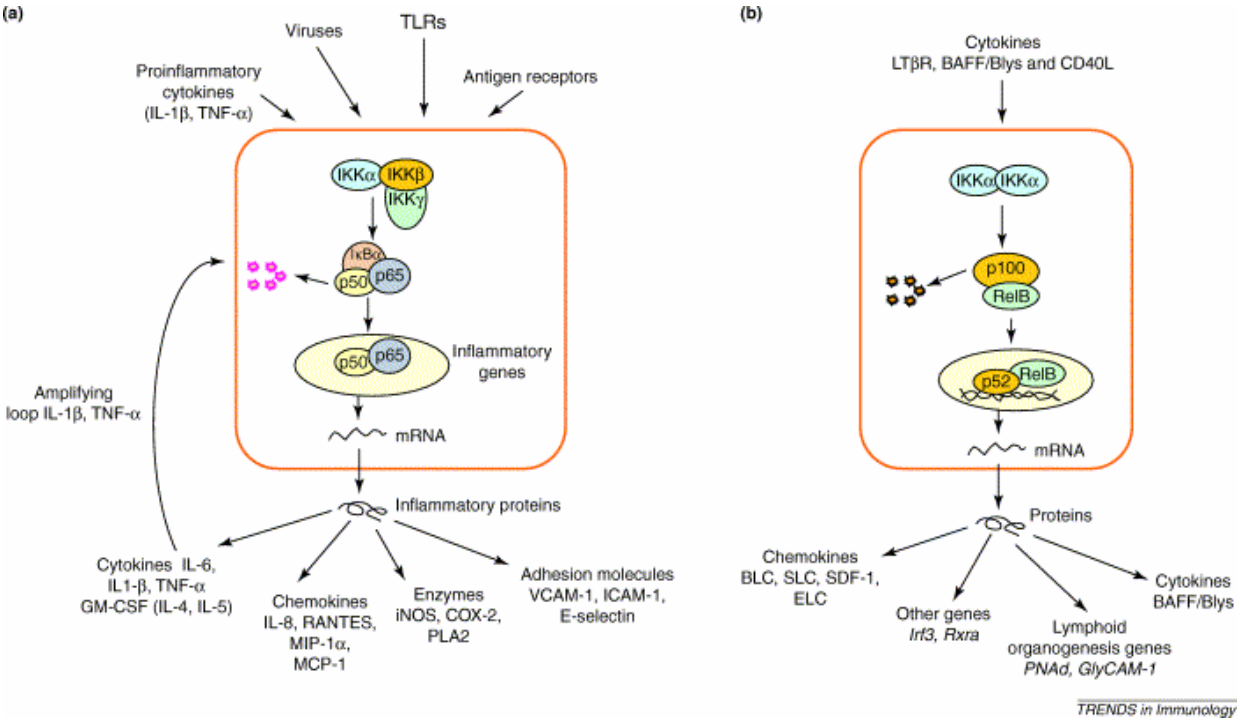


Figure 4. Canonical and non-canonical NF-κB activation pathway. a) Canonical/classical activation ends with the release of the p65/p50 heterodimer into the nucleus; b) Non-canonical/alternative pathway is slower and generates p52/RelB heterodimer. Picture taken from [195].

The canonical pathway is activated by various stimuli such as pathogen-associated molecular patterns (PAMPs) and pro-inflammatory cytokines (IL-1, TNF-α) that act through different receptors such as TNF receptor (TNFR) or Toll-like receptor superfamilies [196]. The canonical pathway is regulated by IKKβ in a NEMO-dependent manner, catalising the phosphorylation of IκBs (at serine residues 32 and 36 in IκBα), lysine ubiquitination (at lysine residues 21 and 22 in IκBα) and degradation by 26S proteosome [177]. This process results in DNA binding and subsequent activation of gene transcription by NF-κB dimmers predominantly formed by p65(RelA), p50 or c-Rel [187]. The canonical pathway mainly activates genes involved in the regulation of the immune system such as cytokines, chemokines, adhesion molecules, enzymes that produce

secondary inflammatory mediators and inhibitors of apoptosis [197]. Knockout mice for IKKβ are associated with an enhanced susceptibility to infections [198, 199].

The non-canonical pathway however is triggered by a subset of stimuli such as BAFF (B-cell activating factor), CD40 ligand or lymphotoxin β (LTβ) in fully developed T and B cells *i.e.* the adaptive immunity [200-203]. Activation of the non-canonical pathway leads to a sustained NF-κB response, involving IKKα activation (independent of IKKβ and NEMO). This leads to the phosphorylation of two C-terminal amino acid residues of p100 and the processing to p52, thereby liberating p52 containing dimmers and leading to nuclear translocation of p52/RelB heterodimmers [199]. Phosphorylation of the precursor form p100/p52 is essential for the processing of p100 to p52 which is also dependent on ubiquitination of the inhibitory C-terminal half of p100 and proteosomal degradation [179].

The non-canonical pathway seems to play a role in the development and maintenance of secondary lymphoid organs. Knockout mice for *Nfkb2*, *RelB*, *IKKα, c-Rel* and *Nfkb1* have an impaired B cell population and proliferation, in the germinal centres of the spleen [199, 204-206].

Alternative pathways that activate NF-κB have also been described, *e.g.* by DNA damaging agents (UV irradiation or doxorubicin) [207, 208].

3. Regulation of NF-κB

The inducible regulation of gene expression is a central element of normal physiology and is the key for a multicellular organism to adapt to environmental, mechanical, chemical, and pathogenic stresses. The diversity of biological roles fulfilled by NF-κB raises several questions about how a limited set of signal transduction molecules regulates signalling to NF-κB in all pathways and, conversely, how discrete inputs create transcriptional responses tailored to specific type of cells, tissues and organs with the same set of regulators. As NF-κB activation can occur by numerous stimuli, triggering transcriptional activation of approximately 500 different genes, a tight regulation of the NF-κB machinery is required [168]. This is achieved by additional regulating levels: a) at the cellular level, *e.g*. differential induction of NF-κB subunits and IκB proteins, recruitment of co-regulators, post translational modifications of NF-κB/IκB/IKK and epigenetics or b) at the tissue or organ level, *e.g*. NF-κB can be regulated by receptor/signalling cascades. All of these layers of regulation are described in the following sections.

3.1. Degradation of specific IκB proteins

Degradation of specific IκB proteins is due to certain signals which also determine the duration of the NF-κB induction [209]. For instance, IκBα degradation is involved in a quick and transient response, whereas IκBβ and IκBε degradation leads to a slow and sustained response [210]. Moreover, distinct IκB proteins associate with a higher affinity with certain NF-κB dimmers (*e.g.* p65(RelA)/c-Rel with IκBε [211]).

3.2. NF-κB dimmers and binding to κB sites

The dimmerisation of the five NF-κB family members in mammals, which results in homo and heterodimmers, leads to a broad spectrum of possibilities for gene targeting and activation. Dimmerisation is a prerequisite for DNA binding. There are 12 activating and 3 repressing dimmers that recognise κB sites [178]. An important feature determining cell and tissue specificity is the fact that the pattern of dimmer formation in different cell types is limited to the specific expression pattern of the NF-κB subunits in each cell type.

The formation of different dimmers and consequently their specific functions in diverse cell types has been described throughout the literature. Homodimmers formed by either p50 or p52 bind to the promoter and repress transcription in unstimulated cells. In contrast, heterodimmerisation of p50 or p52 with a TAD-containing member, will lead to the formation of transcription competent complexes [212]. The heterodimmer formed by p65(RelA) and p50, *i.e.* p65/p50, has a high affinity to κB sites and is very abundant in mammalian cells. As, for instance, in fibroblasts where the majority of TNF-α regulated NF-κB genes are transcribed by p65(RelA)/p50 heterodimmers [213]. Additional examples of cell type or tissue specific NF-κB dimmer patterns include c-Rel and RelB. c-Rel is encountered in haematopoietic and lymphocyte cells. c-Rel knockout mice have been shown to exhibit adaptive immunity as well as innate immune defects in macrophages [214]. As for RelB, which is mainly expressed in lymph nodes, Peyer's patches and certain parts of the thymus, knockout mice have shown not only adaptive immunity but innate immune defects in dendritic cells [215]. This observation emphasises the vast effects of a specific dimmer which goes beyond affecting only a part of a process, in this case, adaptive versus innate immunity.

Another aspect which influences tissue specificity is the formation of specific dimmers at a certain developmental stage. In the case of monocytes and monocyte-derived-macrophages, p50 homodimmers were detected in both unstimulated cells [216]. However, upon LPS stimulation p65(RelA)/p50 and RelB/p50 were only unregulated in macrophages and not in other monocyte-derived cell type.

NF-κB dimmers bind DNA mainly through 10bp long-κB sites with the loose consensus sequence 5'-GGGRNWYYCC-3' (R=purine, W=adenine or thymine, N=any base, Y=pyrimidine) [217, 218]. The κB sites in many promoters are conserved through evolution highlighting the importance of the sequence function for selectivity. The stringency of the dimmer binding to κB sequences and the degree of involvement of the dimmer recruitment is yet not completely clarified.

κB sites are classified according to the bound dimmers: first, class I, for p65(RelA)/p50 and p50/p50 dimmers; and second, class II, where p65(RelA)/p65(RelA) and p65(RelA)/c-Rel bind [219]. Additional evidence highlights the importance of the κB sequence to influence the recruitment of cofactor interacting with a specific dimmer [220, 221]. Together, the degenerate nature of the κB site sequence implies a great sequence variability leading to different binding preferences of the NF-κB dimmers and confers a great polyvalence for the subsequent response [222].

3.3. Transcriptional co-regulators of NF-κB

NF-κB dimmers bind to κB sites within the promoters of target genes and regulate transcription through the recruitment of co-regulators, which include co-activators and co-repressors. Thus, a tight coordination arises between transcriptional co-regulators and NF-

κB. Cell or tissue specificity may be further achieved by expressing co-regulators only in a subset of cells. TAFII105, for example, is a cell-type specific, transcriptional co-regulator confined to B-cell [223].

Co-activators are classified into three groups: a) those modifying histones that regulate DNA access, b) members of the mediator complex which mediate the interaction with the general transcription factor apparatus and RNA polymerase II and c) chromatin remodelling proteins, *i.e*. with DNA-unwinding abilities [224].

Co-activators influencing NF-κB activity include histone acetyltransferases (HATs) like p300/CBP, P/CAF, SRC-1, protein arginine methyltransferase CARM-1/PRMT4, poly(ADP-ribose)-polymerase-1 (PARP-1) and RNA helicase A [212, 225-232]. Co-repressors include for instance histone deacetylases (HDACs) and MYBBP1a [233-235]. HDACs have been reported to form a complex with the p50 homodimmer bound to certain κB-sequences and thereby downregulate gene expression [236]. Acetylation by HATs and deacetylation by HDACs can occur on histone and non-histone proteins. MYBBP1a instead, competes with p300 for the transcriptional activation domain (TAD) of p65/RelA [235]. In fact, expression levels of MYBBP1a depend on the cell type.

Proteins that are usually co-activators can, under certain circumstances, also act as co-repressors and *vice versa*, as it was it has been described for HDACs or p300/CBP [237-238]. Bladder and breast cancer cells treated with various HDACs inhibitors have revealed a comparable number of up- and downregulated genes implying that HDAC activity may not always involve transcriptional repression [237]. p300/CBP can activate reporter gene expression by binding to the transcription activation domain of p65(RelA) [226-227]. However, p300 can also repress transcription *in vitro* using defined specific conditions with

chromatinised templates [238]. Two independent co-regulators may be recruited to a same promoter due to two diverse stimuli. Brg1 or P-TEFb can interact with p65(RelA) upon stimulation with IL-1β or TNF-α, respectively, leading to their simultaneous recruitment to the IL-8 promoter [239, 240].

In addition to traditional co-activator and co-repressor complexes, there is a growing list of proteins that interact with NF-κB dimmers and affect its DNA binding and subsequent NF-κB-dependent transcription [241]. A recent addition is the ribosomal protein S3 (RPS3), which seems to be required for the binding of NF-κB at specific κB sites upon lymphocyte-activating stimuli involved in rapid cellular activation responses [242].

3.4. Post-translational modifications of NF-κB

Transcription of target genes is further regulated through post-translational modifications of the TAD or RHD of NF-κB that affect the ability of NF-κB dimmers to interact with other proteins. Post-translational modifications of the various NF-κB subunits, IκB and IKK proteins described to date, are shown in Figure 3.

The stimulus-dependent phosphorylation of p65(RelA) by protein kinase A (PKA) was the first evidence that NF-κB is subject to post-translational modifications [243]. To date described post-translational modifications of NF-κB include phosphorylation, acetylation, ubiquitination, prolyl-isommerisation and tyrosine nitration [212, 244-246].

There are various phosphor-acceptor sites in the NF-κB subunits. The best characterised are those on p65(RelA) occuring in the RHD (serine residues 276 and 311) and in the TAD (serine residues 529 and 536) [182-185] (Figure 3). Phosphorylation events appear to be critical for the induction of p65(RelA) and to facilitate p300/CBP binding [247]. Serine 276

mapped in the RHD has been found to be phosphorylated by kinase A (PKA) and mitogen- and stress-activated kinase (MSK1 and MSK2) which are activated by LPS or TNF-α, respectively [236, 248]. MSK1 and MSK2 share substrate specificity with PKA. However, phosphorylation itself takes place in different cellular compartments, specifically in the cytoplasm for PKA or at the IL-6 promoter, *i.e.* nucleus, for MSK1.

Protein acetylation is the most abundant protein post-modification in eukaryotes [249]. Acetylation on lysine residues in contrast is less common than in other amino acid residues but has been recently reported for p50 and p65(RelA). The reaction catalised by HATs transfers an acetyl group from the acetyl coenzyme A (acetyl-coA) onto the amino group of a lysine residue [250]. The reaction can be reversed by HDACs, and provides a dynamic response to signalling events. Acetylation of p65(RelA), most likely by p300/CBP and associated HATs, occurs in the nucleus and is associated with an altered transcription [251]. p50 can also be acetylated leading to an increased DNA binding affinity of p50/p50 and p65(RelA)/p50 dimmers [252]. Direct acetylation on p65(RelA) both *in vitro* and *in vivo* has been reported to affect different cellular processes [233, 253, 254]. Acetylation of lysine 122/123 in the RHD of p65(RelA) reduced its DNA binding activity but not its association with IκBα. Thus, acetylation of these lysine residues shortens the length of NF-κB induction. Instead, lysine 221 acetylation increases the affinity for DNA binding and prevents IκBα association, thereby implying a prolonged NF-κB response. Acetylation of lysine 310 has been shown to enhance transcriptional activity without altering the binding to DNA or IκB [247]. Furthermore, p65(RelA) can be acetylated by p300/CBP at lysine residues 314/315 which were reported to repress or activate different genes [255]. Thus, acetylation can contribute to the specificity of the NF-κB response.

Another post-translational modification identified in recent years still under debate, include tyrosine nitration of p65(RelA). Nitration at tyrosine residues 66 and 152, results in p65(RelA) export and rapid inactivation of NF-κB [246].

3.5. Epigenetic regulation of NF-κB

The human body is composed of many different tissues and cells, most of which are terminally differentiated and perform a specific function [256]. These specialised cells can be potentially replaced by a limited pool of stem cells that have the ability to replicate and differentiate into a number of specific cell types [257]. Differentiated cells have a specific phenotype and physiology, leading to a specific pattern of gene expression that arises during differentiation by genomic imprinting including DNA methylation patterns, chromatin remodelling events, heterochromatin formation and formation of special nuclear structures [256]. Therefore, it is important to determine the mechanisms by which NF-κB dimmers interface with chromatin in gene expression.

DNA is packaged into a highly organized and dynamic protein-DNA-complex named chromatin. The nucleosome is the basic subunit of chromatin and is formed by an octamer of four core histones; an H3/H4 tetramer, and two H2A/H2B dimmers, surrounded by 146 bp of DNA [258, 259]. The nucleosome prevents the access of transcription factors and RNA polymerase II them from performing recognition sequences on DNA, therefore imposing a barrier to the initiation of transcription. A major mechanism of transcription regulation is the alteration of the chromatin structure surrounding genes and their regulatory elements. The chromatin structure confers cell type specific positioning of a chromosome within the nucleus, which can affect its heterochromatin status [260].

Specific cell localisation of genes within chromatin territories determines the potential activity of these genes. For instance, genes located on the outside the chromatin territory are more active than those within the territory or at the nuclear periphery [256]. This allows the repression of a vast number of genes, whose protein products are not necessary in a specifically differentiated cell. NF-κB-dependent genes are divided into genes containing: a) constitutively and immediately accessible promoters that do not require chromatin modification and b) late accessible promoters that are dependent on stimulus-induced chromatin modification [261]. The local chromatin structure varies from one cell type to another as the cells possess different patterns of histone modifications and positioned nucleosomes in a certain region of specific genes. A well documented example of the direct regulation of local chromatin to the transcriptional potential of p65(RelA) is the chromatin surrounding the E-selectin promoter [262]. E-selectin is an adhesion protein expressed only in endothelial cells and is involved in the binding of leukocytes at sites of infection and inflammation. Expression of E-selectin was reported to be regulated by NF-κB and pro-inflammatory cytokines. When the proximal promoter of E-selectin was used in reporter assays, it was activated in non-endothelial cells, which implies that the endogenous E-selectin expression is regulated by its endogenous status.

Moreover, the pattern of heterochromatin, *i.e.* the form of chromatin which is highly condensed throughout the cell cycle, varies within an organism among its cell types and allows the repression of genes that are not necessary for the specialised function of a differentiated cell [263]. In contrast to differentiated cells with a specific pattern of heterochromatin, stem cells have little heterochromatin, thereby allowing multiple potential differentiation pathways [260]. Cell type specific heterochromatin and chromatin structure

are likely to have a large role in the regulation of NF-κB dependent transcription but further research is required.

3.6. Receptors/Signalling cascades

A broad spectrum of stimuli ranging from endogenous, exogenous, physical and chemical stresses lead to the activation of NF-κB. Cell and tissue specificity is greatly dependent on the cell surface receptors and signal transduction molecules that are specific to a determined type of cell or tissue. An example of cell specificity through cell surface receptors are CD14 and TLR4, which are exclusively found in myeloid cells. Briefly, LPS binds to CD14, activating Toll-like receptor-4 (TLR-4) and downstream signalling pathway as well as transcriptional factors that regulate the expression of many different inflammatory cytokines [264]. Therefore, cell receptor specificity of CD14 and TLR-4 implies that LPS can activate NF-κB only in these cells.

The recognition of stimuli is achieved by receptor-ligand. TLRs is a family of proteins shared between different stimuli in the canonical pathway. However, depending on the specific ligand, a particular TLR is activated. This enables the innate immunity to deal with a wide diversity of PAMPs. TLR2 recognises a spectrum of microbial products such as peptidoglycans and lipoprotein, produced by Gram positive bacteria. However, TLR3, TLR4, TLR5 and TLR9 recognise other PAMPs such as double stranded RNA produced by viruses, LPS from Gram negative bacteria, bacterial flagelling, and non-methylated bacterial DNA, respectively [265-268]. Ligand binding to all of these TLRs leads to the activation of NF-κB and subsequent induction of target genes [269].

The TNF receptor-associated factor (TRAF) family of proteins constitute key intermediates in both, the canonical and non-canonical NF-κB signalling pathways [270-272]. Different TRAF family members are recruited intracellularly upon distinct stimulation. In the canonical pathway, distinct TRAFs are recruited depending on the activated receptor. TRAF2 and TRAF5 are both required for NF-κB activation through TNFR1, TRADD, and TNF-α [273-275]. Conversely, in Toll/IL-1 signalling TRAF6 is recruited by the receptor complex and is necessary for MyD88-dependent activation by IL-1 and ligands of TLR4 [184, 276]. In the non-canonical pathway, TRAF3 is normally recruited by the receptor upon stimulation, and undergoes signal-dependent degradation leading to NIK accumulation and activation of NF-κB gene expression [277].

4. Aim of the thesis

Age-related (degenerative) changes of the IVD occur on the extracellular matrix and on the phenotype of IVD cells. A key factor for these alterations is a compromised disc nutrition which occurs with ageing. Moreover, oxidative stress in the IVD leads to the appearance of advanced glycation end products (AGEs). Cellular stress can also lead to the formation of highly damaging oxidants (*e.g.* peroxynitrite) which induce tyrosine nitration. These alterations can potentially lead to the activation of NF-κB, a transcription factor that regulates the expression of a large variety of genes involved in pro-inflammatory responses. Pro-inflammatory mechanisms are thought to play a decisive role in the degenerative changes of the IVD and possibly link to the appearance of subsequent pain.

The aim of this thesis was therefore to investigate the underlying signalling cascade, casually linking age-related cellular- and extracellular- post-translational alterations to a NF-κB-dependent pro-inflammatory pathway. As a response, the released cytokines may either directly or indirectly (*e.g.* via neurothrophic factors) cause nociception at the outer annular disc fibres resulting in discogenic back-pain.

RESULTS

5. Research articles

5.1. Role of N-(carboxymethyl) lysine modifications in NF-κB translocation and MMP-13 gene expression in bovine nucleus pulposus cells.

Submitted manuscript

5.2. Peroxynitrite induces gene expression in human intervertebral disc cells.

Submitted manuscript

My contribution to both papers was the conception and performance of all the experiment and the write up of the manuscripts.

TITEL: Role of N-(carboxymethyl) lysine modifications in NF-κB translocation and MMP-13 gene expression in bovine nucleus pulposus cells.

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Abstract

Study design: Bovine nucleus pulposus (NP) cells were cultivated in monolayers and stimulated with advanced glycation end products (AGE) or N-(carboxymethyl) lysine (CML). AGE receptor expression, NF-κB activation, MMP13 and cytokine profiling were analysed.

Objective: To determine the effect of AGE/CML on NP cells.

Summary of Background Data: Age–related accumulation of AGE as CML has been reported in human intervertebral discs (IVD). AGE/CML can be recognised by various receptors; ligand binding to the receptor can potentially lead to activation of NF-κB with induction of a pro-inflammatory cascade as well as influencing tissue remodelling by activation of proteinases.

Methods: NP cells with pericellular matrix ("chondrons") were isolated from bovine coccygeal IVD by partial digestion to avoid disruption of the cell. NP cells were grown in monolayers and expanded in media DMEM/F12 containing low glucose and serum levels to resemble the conditions encountered in a normal intervertebral disc (IVD). AGE or CML modified BSA was used to stimulate NP cells. The AGE receptor expression and levels of the pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) were monitored by RT-PCR or quantitative RT-PCR, respectively. NF-κB/p65 subunit translocation into the nucleus and MMP13 expression were analysed by immunoblotting.

Results: Neither AGE nor CML-BSA induced changes in the gene expression of cytokines nor in the AGE-receptors analysed in this study. However, NF-κB/p65 subunit was translocated into the nucleus of bovine NP cells on stimulation, indicating that AGE/CML activated NF-κB. Furthermore, CML-BSA increased protein levels of the MMP-13 pro-form.

Conclusion: Our data suggest that the expression of the examined AGE receptors were not affected by its ligands AGE/CML. However, AGE/CML did activate NF-κB and the NF-κB-dependent proteinase MMP-13, implying that the accumulation of AGE in the matrix affected tissue remodelling and/or disruption.

Key words: Advanced glycation end products (AGE), N-(carboxymethyl)lysine (CML), NF-κB, MMP-13, disc degeneration, low-back pain, discogenic pain, intervertebral disc

Key points:

- AGE and CML *i.e.* non-enzymatic glycoxidation protein modificatons lead to NF-κB activation in a bovine chondron culture model.
- No significant effect of AGE or CML on the pro-inflammatory cytokines: IL-6, IL-1β and TNF-α.
- CML modulates MMP-13 expression indicating a putative role in disc matrix remodelling.

Mini Abstract/Precis: Age–related accumulation of advanced glycation end products (AGE), *i.e.* N-(carboxymethyl)lysine (CML), have been reported in human intervertebral discs (IVD). The bovine chondron model has enabled the study of the effects *in vitro* of AGE/CML showing an NF-κB dependent pathway and modulating MMP-13 expression.

Introduction

Disc degeneration is an age-related process which can start as early as the second decade of life [1]. The compromised disc nutrition at the end of the second decade of life appears to be key in initiating disc degeneration [2]. Physiological alterations of the intervertebral disc (IVD) involve a limited supply of oxygen and nutrients, *i.e.* glucose, as well as an acidic pH due to lactate accumulation [3-7]. However, the molecular pathways connecting the changes in the environment of the IVD cells to the matrix alterations and possible breakdown remain widely unknown. These alterations include loss of proteoglycans from the nucleus pulposus, degradation of matrix components by locally expressed proteinases and a reduction of the number of disc cells (reviewed in [8,9]). Matrix proteins present in the extracellular matrix (ECM) of the IVD can also be modified by post-translational modification. Post translational non-enzymatic glycation, known as Maillard reaction, results in the formation of various species of advanced glycation end products (AGE) such as N-(carboxymethyl) lysine (CML), N-(carboxyethyl) lysine (CEL) and pentosidine [10-13]. CML is one of the best-characterised AGE-species and is considered a biomarker for enhanced or accumulated oxidative stress in the respective tissue [14]. Immunolocalisation of CML in the IVD, revealed age-dependent CML deposition in areas of macroscopic and histological degeneration of the IVD matrix [15]. This is in line with age-dependent accumulation of CML in other tissues consisting of long-lasting matrix proteins [16-21]. Accumulation of CML and other AGE can be recognised by AGE-binding receptors such as the multi-ligand receptor RAGE (receptor for advanced glycation end products) and the components of the so-called AGE-receptor complex comprising AGE-R1/oligosaccharyltransferase (OST)-48, AGE-R2/80K-H as well as AGE-R3/galectin-3 [22-24]

. For instance, ligand-binding of RAGE or AGE-R2 have been shown to activate key signalling pathways comprising NF-κB, which can potentially result in a proinflammatory signalling cascade involving pro-inflammatory cytokines [25,26].

We hypothesise that accumulation of CML in the ageing IVD matrix might initiate a signalling pathway involving NF-κB with resulting consequences to a range of cellular functions including the expression of pro-inflammatory cytokines. Alternatively, accumulation of AGE/CML on long lasting matrix proteins might be involved in tissue remodelling, *e.g* potentially disrupting protein folding, protein-protein interactions with a subsequent brittleness of the extracellular matrix. Matrix metalloproteinase (MMP)-13, a collagenase 3, is a candidate proteinase involved in tissue remodelling that can be regulated by NF-κB [27-29] and is one of the main proteinases responsible for cleaving collagen type II, which is highly abundant in the disc [30-31].

In using a bovine chondron model[32-33], the aim of this study was to examine the effects of AGE (CML) on bovine NP cells by investigating i) NF-κB activation, ii) the regulation of matix degrading enzymes (e.g. MMP-13) and iii) the expression of pro-inflammatory cytokines which may play a role in the generation of so-called discogenic back-pain [34-37].

Material and methods

Cell Isolation. Bovine coccygeal spines (n>50) were obtained from the local abattoir (age approx. 2 years) and processed within 3 hours of death. The discs were dissected and the nucleus tissue carefully separated from the annulus. Discs with damage to the adjacent bony endplate were excluded. Nucleus pulposus cells with their pericellular matrix were isolated

by 0.2% collagenase IA (Sigma) / 0.3% dispase II (Roche, Applied Science) digestion (w/v in PBS). OK medium consisted of 50 % DMEM without glucose (Gibco) and 50 % F12, 2 % FBS, 1% ITS (Insulin, Transferrin, Selenium; Sigma), and 1 % antibiotic/antimycotic (penicillin, streptomycin, amphotericin B; Gibco). Digestion was carried out overnight at 37° C (100 % humidity, air, 5 % CO_2). Digested tissue and medium were filtered with a 70 μm cell strainer (Falcon) to remove undigested fragments, washed three times with OK medium and resuspended in OK medium. Viable cells, as determined by Trypan blue exclusion, were counted and transferred to a 10cm dish. The cultures were incubated at 37° C (100 % humidity, air, 5 % CO_2). The medium was changed after 1 day and every three days thereafter. Bovine NP cells were used up to passage 4 in all of our experiments.

Antibodies. Rabbit polyclonal antibody to collagen type VI (Acris Antibodies GmbH), rabbit polyclonal antibody to NF-κB/p65 (C-20, Santa Cruz Biotech), rabbit polyclonal antibody to N-(carboxymethyl)-lysine (CML) (Dr. E. Schleicher, Tuebingen, Germany), goat antibody to RAGE (N-16, Santa Cruz Biotech, Inc.), mouse monoclonal antibody to the proliferating cell nuclear antigen (PCNA) (PC-10, Santa Cruz Biotech, Inc), goat polyclonal antibody to AGE-R2/Glucosidase II (N-19, Santa Cruz Biotech, Inc.), rabbit polyclonal antibody to AGE-R1/OST-48 (H-300, Santa Cruz Biotech, Inc), rat hybridoma antibody to AGE-R3/galectin-3 (Prof. A.W. Stitt, Belfast, UK), rabbit polyclonal antibody to MMP-13 (Chemicon/Millipore), goat anti rabbit Alexa Fluor 488 antibody (Molecular Probes), peroxidase labelled anti mouse/rabbit/rat or goat antibody (Amersham Biosciences).

Immunohistochemistry. Pepsin-mediated antigen retrieval (Pepsin Solution, Lab Vision Corporation) was performed on 5 μm tissue sections at 37°C for one hour and then rinsed in PBS. The slides were blocked in 1 % BSA (Sigma), 0.2 % dry milk powder and 0.1 % Tween 20 in PBS for one hour, followed by the incubation of a 1:100 dilution of the primary antibody in 1 % BSA and 0.1 % Tween 20 in PBS for one hour. Tissue sections were rinsed in PBS and incubated for a further 30 minutes with a 1:400 dilution of the secondary antibody in 1 % BSA and 0.1 % Tween 20 in PBS. The slides were rinsed again in PBS and counterstained with DAPI (1:1000) in PBS for 15 minutes. Sections were rinsed, mounted with Vectashield (Vector Lab, Inc, CA) and viewed under the fluorescent microscope.

AGE-BSA preparation. 50 mg BSA Fraction V (Sigma, A-9543) were incubated with 50 ml PBS, pH 7.4, and 750 mg of ribose (Sigma) for 5 weeks at 37° C and 5 % CO_2 to allow AGE accumulation. The solution was dialysed against PBS in two cycles of 6 hours to eliminate the ribose.

CML-BSA preparation. 40 mg BSA Fraction V (Sigma, A-9543) and 40mg Glycoaldehyde Dimer (Fluka 50580) were dissolved in 20 ml 0.5 M Na-phosphate buffer, pH 7.4, to obtain a concentration of 2 mg/ml. The CML-BSA solution was incubated for 3 days at 37° C and 5 % CO_2 to allow CML accumulation. The concentration of each of the modified BSA preparations used to stimulate bovine NP cells for all experiments was 200 mg/ml.

Conventional RT-PCR. Total RNA from bovine NP cells and native tissue was isolated with the Qiagen RNeasy kit (Qiagen, Germany) and reverse transcription was performed using the RT-Statascript kit according to manufacturer's protocol. For RNA isolation of

native tissue; this was snap frozen in liquid nitrogen, pulverised and immediately processed. Primer Pairs were designed with the Light-Cycler Probe Design Software 2.0 (Roche Diagnostics, Switzerland). The sequences of the bovine primers were: *GAPDH 5'* TCCGGGAAGCTGTGGCGT *3'* AGGCGGCAGGTCAGATCCACAA, *MMP-2 5'* GGAACAGATCACATACAGG *3'* CACCAAAGGAAGCCATCG, *HIF-1α 5'*GAGGTGGATATGTCTGGATAGAAACTCAAG *3'*AACTGGTTTGAGAACACATTCTGTTTGTTGAAG, *collagen type II 5'* ATCCATTGCAAACCCAAAGG *3'* CCAGTTCAGGTCTCTTAGAG, *collagen type VI 5'* CGCTGTGCAACCACGAA *3'* TCCATCTGTTTGGCAGGG,

Briefly, denaturation at 94˚ C for 3 minutes, amplification of 30 cycles with denaturation at 94˚ C for 40 seconds, primer-dependent annealing temperature for 40 seconds and elongation at 72˚ C for 20 seconds. All PCR products were resolved by 1.5 % agarose gel electrophoresis, and the DNA bands were visualised by staining the gel with ethidium bromide. Semi-quantification was performed using a Versa-Doc ^{TM} Imaging System (Bio-rad). The values were normalized relative to the reference gene GAPDH to compensate for differences in cDNA loading.

Nuclear and Cytoplasmic Extract preparation and immunoblotting. Nuclear and cytoplasmic extracts and immunoblotting were performed as described by Hottiger *et al*. (1998).

Quantitative PCR (RT-qPCR). Total RNA from bovine NP cells was extracted with the RNA isolation kit from Agilent according to the manufacturer's instructions. cDNA was synthesized from 10 ng of total RNA using High Capacity cDNA Archive Kit (Applied

Biosystems). The cDNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (Customed TaqMan Gene Expression Assays, Applied Biosystems). All samples were biological triplicates run in duplicate. Relative expression levels were determined using a four-point serially diluted standard curve generated from cDNA from bovine NP cells. Expression levels were expressed in arbitrary units, setting the untreated value as 1. The values were normalized relative to the reference gene S18 protein mRNA to compensate for differences in cDNA loading.

Results

Isolation of bovine nucleus pulposus-derived chondrons

First, we determined the feasibility of isolating NP cells with their PCM. According to the terminology, in cartilage, cells plus PCM are termed chondrons. All discs used in this study were morphologically very similar, with a white NP that was clearly distinguishable from the annulus fibrosus. Morphology of the IVD was comparable to grade II human discs according to Thompson's scale [39]. Digestion of NP tissue with 1 % collagenase-only or with a mixture of 0.3 % dispase/0.2 % collagenase (adapted from Larson *et al.*, 2002) led either to preparations containing mostly chondrons or to chondrocyte-like single cell preparations, respectively (Fig. 1B and C). The presence of the pericellular matrix was confirmed by immunolocalisation of collagen type VI, which has been shown to be preferentially localised in the PCM [41]. Viability of the cells was always higher than 90 % as determined by Trypan blue exclusion. Comparisons between chondron suspension and native NP tissue revealed a very similar distribution and expansion regarding collagen type VI positive

matrix (Figure 1A and 1B). The immunofluorescence data was further corroborated by RT-PCR for collagen VI (Figure 1D, lanes 4 and 8). This suggests the presence of the PCM in the chondron suspension used for subsequent monolayer culture.

Additional phenotypical characterization of the NP chondrons was performed using RT-PCR for matrix metalloproteinase (MMP)-2, hypoxia inducing factor (HIF)-1α and collagen type II. These markers were present in chondrons (Figure 1D, lanes 1-3) as well as in the control, native NP tissue (Figure 1D, lanes 5-7). Additionally, the phenotypic characterisation of bovine chondrons expanded in a monolayer at passage 4 also showed comparable mRNA levels to freshly isolated chondrons (data not shown).

AGE-receptor profiling on AGE/CML-BSA stimulation

To determine which AGE-receptors are expressed and whether their expression is affected by AGE or CML treatment, chondrons were stimulated for 24 or 48 hours with AGE/CML-BSA and analysed by immunoblotting (Figure 2). To ensure that the receptor profile was not disrupted or modified during the enzymatic isolation, it was monitored on native NP tissue and freshly isolated chondrons using conventional RT-PCR (data not shown). The AGE receptor complex components were present in the native tissue whereas RAGE could not be detected in neither samples, at mRNA or at protein level (data not shown). As a positive control for RAGE, bovine lung tissue was used. There were no differences between stimulation for 24 (Figure 2) and 48 hours (data not shown). For AGE-R3 two distinct bands could be detected at approximately 30 and 70 kDa (as indicated by the arrows in Figure 2), which would correspond to the monomer and dimmer, respectively. In addition, the presence of the several receptors for AGE was monitored in human biopsies

of pathological IVDs by RT-PCR. The data corroborated the bovine data on the presence of the AGE receptor complex units in the human IVDs (data not shown).

NF-κB subunit p65 translocation into the nucleus

To study the translocation of NF-κB into the nucleus upon stimulation with AGE/CML-BSA, nuclear and cytoplasmic extracts were prepared for samples stimulated with AGE-BSA or CML-BSA for 20 minutes (Figure 3A, top panel). PCNA served as a loading control for the nuclear extracts, Figure 3A, lower panel. In untreated NP cells no NF-κB induction, *i.e.* translocation into the nucleus, was observed. Induction of NP cells with AGE and CML-BSA lead to NF-κB/p65 translocation into the nucleus. 1 μg/ml TNFα treatment served as a positive control for NF-κB/p65 translocation into the nucleus, which is indicative of its activation.

Effects of AGE/CML-BSA on MMP-13 gene expression

To determine whether AGE or CML have an effect on MMP-13 gene expression, MMP-13/collagenase 3 was monitored upon the stimulation with AGE/CML-BSA. At 6 hours MMP-13 was strongly induced, in its pro-form, upon CML- BSA (Figure 3B). Again, bovine lung tissue served as a positive control for MMP-13 in both forms the pro and mature form, 60 and 48 kDa bands respectively.

Pro-inflammatory cytokines in AGE/CML-BSA signalling pathway

Real-time PCR enabled the quantification of pro-inflammatory cytokines that could be involved in disc degeneration; these included, IL-6, TNF-α and IL-1β (Figure 4). Chondrons were stimulated for 3 or 6 hours under conventional culturing conditions. Despite being present in all cultures at basal levels, neither TNF-α, IL-6 nor IL-1β gene expression levels varied significantly upon AGE-BSA or CML-BSA treatment. TNF-α stimulation was performed as a positive control for the induction of IL-1β and TNF-α gene expression whereas LPS stimulated IL-6 mRNA levels.

Discussion

The source and origin of discogenic back-pain are as yet widely unknown. However, there is a consensus that degenerative changes in the matrix of the IVD are of primary importance [15, 42-45] , even though the link between morphological alterations in the matrix and pain induction is still unclear. Recently, there has been a growing interest in the expression of cytokines in the disc matrix that might have contributed to degeneration or pain induction [34-37] as well as disruption in the levels of proteolytic enzymes, which are in charge of the maintenance of tissue homeostasis [45-47]. Post translational non-enzymatic modifications such as advanced glycation end products (AGE) also result during the processes of ageing and degeneration [15-21]. In a histological study, Nerlich *et al.* have observed that post-translational protein modifications such as for example the irreversible formation of CML in collagen fibers accumulate with progressive disc degeneration [15]. AGEs can be recognised by several receptors for AGE and ligand binding can lead to activation of NF-κB with consequences to a range of cellular functions, including the

induction of pro-inflammatory cascades [23, 48] and matrix metalloproteinases. These findings suggest that CML or AGE accumulation might be the starting point of a signalling cascade, connecting age-dependent matrix alterations to the expression of matrix degrading proteinases. Expression of the cytokines might then lead to pain induction responsible for discogenic low back-pain.

Investigations on molecular signal transduction events leading to CML accumulation in the disc *in vivo* is hampered by the limited possibilities of influencing the conditions inside the IVD. In this study, a monolayer culture system based on easily accessible bovine IVD NP cells was used as it has been shown to be phenotypically similar to human NP cells [32]. To minimise the disturbances to the cells, NP cells were isolated as 'chondrons' [33, 40], *i.e.* IVD cells covered by their pericellular matrix. As high glucose levels are not present in the *in vivo* situation and might lead to artificially high levels of glycated matrix proteins *in vitro*, glucose levels were reduced to 5 mM and fetal calve serum was reduced to 2 % with the addition of 1 % ITS. ITS as a serum replacement, has been successfully used in articular cartilage as it promotes a reduced chondrocyte differentiation and higher quality of tissue formation [49-50]. Phenotypic stability of the isolated disc cell chondrons expanded as a monolayer cultures was assessed throughout the experiments. The presence of the PCM and success of our isolating method was determined by collagen type VI. In addition, phenotypic stability was examined in freshly isolated as well as expanded chondrons; passage 4 shared the same phenotypical properties of the native tissue like disc cell markers such as MMP-2, HIF-1α and collagen type II. Taken together, the data suggest that the chondron-based system might be a suitable model to culture disc cells *in vitro*.

Identification of the receptors involved in AGE-ligand binding in the IVD was analysed after stimulation of cultured chondrons with either AGE- or CML-BSA. A screening was performed using immunoblotting and conventional RT-PCR by examining the receptor for AGE (RAGE), and the components of the so-called AGE-receptor complex. However, expression at gene and protein level of RAGE could NOT be detected either in the bovine chondrons or in the native bovine tissue. To avoid any species-specific differences between the bovine cells applied in this study and human IVD cells, human IVD biopsies were assessed in parallel. The presence of RAGE mRNA could not be detected in any of the 6 human degenerated intervertebral biopsies despite being present in the human lung as analysed by RT-PCR (data not shown). The discrepancy of our results with other studies [26, 51] with regard to the absence of RAGE in human degenerated IVD should be further analysed and immunohistochemical studies interpreted with care. Additional AGE receptors that might substitute for RAGE have been described recently. The so-called AGE-receptor complex was first described by Li *et al.* in 1996 and consisted of AGE-R1 / OST-48, AGE-R2 / 80K-H and AGE-R3 / galectin-3. The subunits of the AGE-receptor complex were present in disc chondrons (Figure 2) and native tissue (data not shown) but were not induced on stimulation by AGEs as no changes were detected at protein level compared to the basal level before stimulation. Interestingly, two bands were detected for AGE-R3, most likely representing the monomer and dimer form of AGE-R3 as previously published [52].

Our results indicate that an NF-κB-dependent pathway is indeed activated as NF-κB/p65 was translocated into the nucleus on AGE/CML-BSA stimulation. To investigate if glycated proteins do have the ability to induce the expression of potentially NF-κB-dependent proinflammatory cytokines, expression levels of IL-6, IL-1β and TNF-α were

analysed after incubating chondrons to AGE- or CML-modified proteins (BSA). Surprisingly, neither exposure to AGE- or CML-BSA did significantly affect the gene expression levels of the investigated cytokines. Only mild effects such as one fold increase upon stimulation for IL-6 and IL-1β were observed. This is in contrast to previous studies revealing that AGE/CML are potent inducers of a proinflammatory cascade in other cell types [25-26].

Contrary to the findings regarding cytokine expression and AGE-receptor activation, the nature of the advanced glycation end products does seem to influence differently tissue remodelling processes by modulating MMP expression. MMPs have been reported to be important factors in the catabolic processes involved in matrix turnover in the IVD. Homeostasis is a key feature in this turnover between matrix proteins, matrix degrading proteins and their inhibitors. MMP-13 or collagenase 3 was analysed as it can be regulated by NF-κB [27-29] and is one of the main proteinase responsible for the degradation of collagen type II [30, 31]. Our results indicate that CML-BSA upregulates the protein levels of the pro-form of MMP-13, which is active but can be cleaved to a more active mature form when released into the ECM. An upregulation of MMP-13 would then tip the balance to catabolic processes severly affecting tissue turnover.

Clinical relevance. The presence of glycated proteins did not stimulate the expression of pro-inflammatory cytokines or affect the expression or activation of putative receptors for the presence of AGE. However, the activation, possibly via NF-κB, of a major proteinase involved in tissue remodelling of the IVD matrix suggests an important role for AGE modified proteins during degeneration of the IVD.

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Legends

Figure 1. Phenotypical Characterisation of the bovine native nucleus pulposus tissue and chondron suspensions derived from bovine NP. Immunolocalisation of collagen type VI in native bovine NP tissue

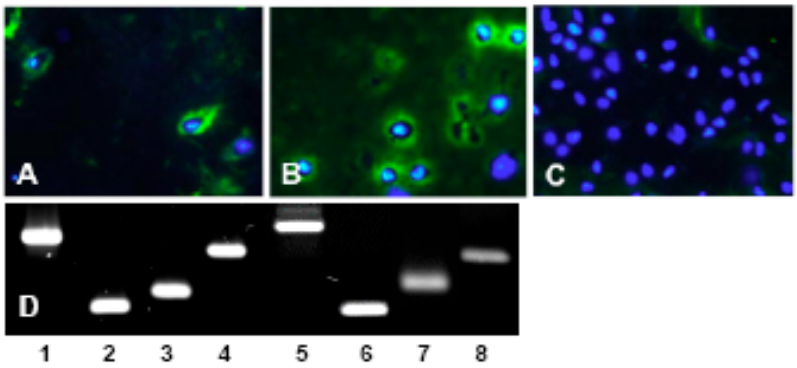
(A), NP-derived cell *(B)* or NP-derived chondron *(C)* suspensions (blue = nuclear stain with DAPI, green = collagen type VI). Original magnifications were 100 x for *A* and 40 X for *B* and *C*. Characterisation using RT-PCR of the native bovine NP tissue and the bovine chondron suspension *(D).* Columns 1-4 and 5-8 correspond to native NP tissue and chondron suspension, respectively. Columns 1 and 5 correspond to MMP-2, 2 and 6 to HIF-α, 3 and 7 to Collagen type II and 4 and 8 to Collagen type VI.

Figure 2. Expression of AGE receptors upon stimulation with AGE/CML-BSA. Bovine chondrons were stimulated with either AGE or CML-BSA for 24 hours. Immunoblotting was performed to detect the presence of AGE-R1/2/3 and RAGE in bovine NP cells. Bovine lung tissue served as a positive control for RAGE.

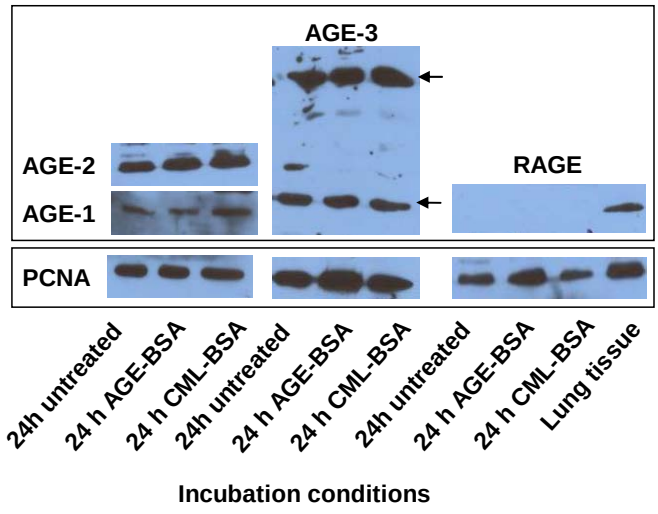
Figure 3. p65NF-κB translocation into the nucleus and MMP-13 expression in bovine nucleus pulposus cells stimulated with AGE/CML-BSA. Panel *A* corresponds to p65NF-κB translocation into the nucleus of bovine NP cells. The nuclear extracts were analysed by immunoblotting. UT stands for untreated. TNF-α (1μg/ml) stimulated cells served as positive control. PCNA served as a loading control. This panel represents three independent biological replicates (n=3). Panel *B* corresponds to Western blot analysis of whole cell extracts of bovine NP cells stimulated with either AGE-BSA or CML-BSA for 6 hours to detect MMP-13. Bovine lung tissue served as a positive control. The molecular weight of the precursor and mature form of MMP-13 are 60 and 48 kDa, respectively. This panel represents three independent biological replicates (n=3).

Figure 4. Pro-inflammatory cytokine profile of bovine nucleus pulposus cells stimulated with AGE/CML-BSA. All experiments were performed under normal culturing conditions. The cytokine profile for IL-6, IL-1β and TNF-α was analysed using quantitative real-time RT-PCR. NP cells were stimulated with either AGE/CML-BSA. TNF-α and LPS stimulated cells served as a positive control showing that the above mentioned cytokines can be indeed induced. On the *y* and *x* axis the fold increase and the time point in hours is represented, respectively. Expression levels were expressed in arbitrary units, setting the untreated value as 1. The values were normalized relative to the reference gene S18 protein mRNA to compensate for differences in cDNA loading. This figure represents a mean of at least three independent biological samples.

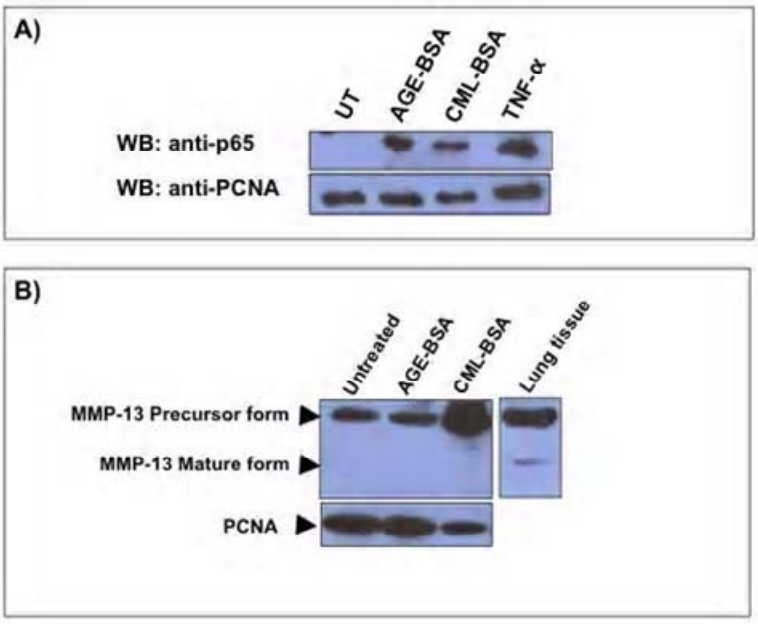
Poveda, *et al*.,Figure 1



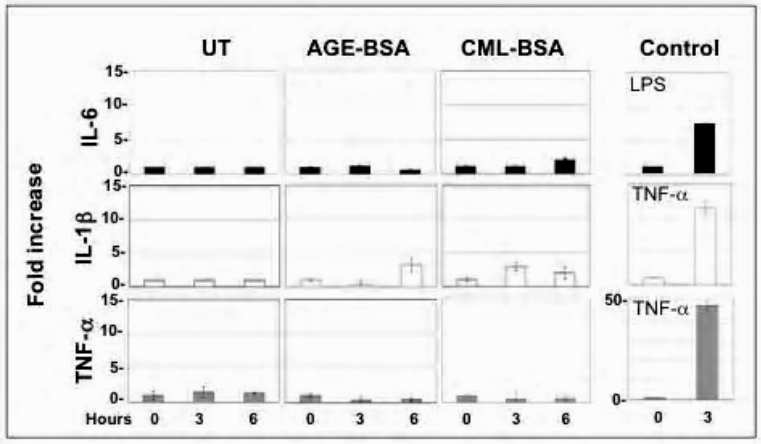
Poveda, *et al*.,Figure 2



Poveda, *et al*.,Figure 3



Poveda, *et al*.,Figure 4



Peroxynitrite induces gene expression in intervertebral disc cells

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This study was supported by AOSPINE, SWITZERLAND
(SRN 02/103 and AOSBRC-07-03)

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Peroxynitrite induces gene expression in intervertebral disc cells.

Abstract

Objective: To investigate the oxidative/nitrosative effects of peroxynitrite on human nucleus pulposus cells.

Background Data: Peroxynitrite is an important tissue-damaging species generated at sites of inflammation and degeneration. The aim of this study was to examine the effects of oxidative/nitrosative stress caused by peroxynitrite and the peroxynitrite donor SIN-1 in human nucleus pulposus cells.

Methods: Degenerated human intervertebral disc tissue was analysed for nitrosylation by immunofluorescence. In addition, human nucleus pulposus cells were isolated from intervertebral discs, expanded and stimulated either with peroxynitrite itself or a stable peroxynitrite donor (SIN-1). Nitrosylation, accumulation of intracellular reactive oxygen species, NF-κB nuclear translocation and cell viability were analysed by fluorescence. Gene expression of TNF-α, IL-1β, IL-6, IL-8 and IL-10 was quantified by real-time RT-PCR.

Results: Degenerated intervertebral disc tissue showed strong nitrosylation, especially in the nucleus pulposus. Isolated human nucleus pulposus cells showed a strong signal for nitrosylation and intracellular ROS upon stimulation with peroxynitrite or SIN-1. NF-κB/p65 sustained nuclear translocation of NF-κB/p65 and stimulation of IL-1β, IL-6 and IL-8 expression was noted upon treatment of cells with SIN-1.

Conclusions: This study provides evidence that peroxynitrite may play a role in disc degeneration by an increased synthesis of pro-inflammatory cytokines. Nuclear

translocation of NF-κB was identified as the potential underlying pathway. Thus, neutralising peroxynitrite and its derivatives (*e.g.* via the use of antioxidants) may be a novel treatment approach to deal with inflammatory processes and discogenic degeneration.

Key words:

Intervertebral disc, degeneration, peroxynitrite, SIN-1, NF-κB activation, tyrosine nitration, pro-inflammatory cytokines.

Key points:

1. Tyrosine nitration due to oxidative/nitrosative stress is present in the human degenerated intervertebral disc.
2. Peroxynitrite is a highly reactive agent formed *in vivo* from the interaction of superoxide and NO.
3. Peroxynitrite and SIN-1 (a stable peroxynitrite donor) induce nitrosylation in NP cells as well as the activation of an NF-κB-dependent signalling pathway, resulting in the upregulation of pro-inflammatory cytokines.

Mini abstract:

The presence of nitrosylation in degenerated intervertebral discs suggests a role of reactive oxygen/nitrogen species in the process of disc degeneration. In human nucleus pulposus

cells, peroxynitrite and SIN-1 stimulated expression of pro-inflammatory cytokines which correlated with sustained nuclear translocation of NF-κB.

INTRODUCTION

Disc degeneration is an age-related process which can start as early as the second decade of life [1]. It is widely accepted, although not conclusively proven, that the level of oxidative stress increases with ageing, possibly by inducing damage of macromolecules and disrupting the homeostasis between reactive oxygen and nitrogen species production and anti-oxidant defences. This sets the basis of the 'free radical theory of ageing' where organisms 'age' due to the accumulation of free radical damage as time passes [2, 3]. Moreover, a variety of inflammatory mediators have been implicated in intervertebral disc (IVD) degeneration or pain induction such as the expression of cytokines in the disc matrix [4-7]. Special attention however, has recently been drawn towards reactive oxygen and nitrogen species for their role in disc degeneration and sensitisation of dorsal root ganglion neurons [8, 9]. While free radical species have been shown to contribute to vital cell signalling mechanisms, there is evidence for a detrimental effect of these highly reactive and labile molecules in tissues such as cartilage with their implication in cell senescence [10-12], inhibition of proteoglycan synthesis as well as general degeneration of the extracellular matrix, *i.e.* damage of structural ECM proteins and activation of ECM proteinases [13, 14]. Furthermore, in degenerated cartilage, reactive oxygen and nitrogen species have been shown to correlate with high levels of pro-inflammatory cytokines such

as IL-1β [15]-[16]. Several reports have investigated the presence of free radical components such as nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in the degenerating and herniated disc [5, 17]. Using a cell culture model, human nucleus pulposus (NP) cells have been shown to produce RNS [8, 18].

Peroxynitrite is a highly reactive agent formed *in vivo* from the interaction of superoxide and NO [19]. Peroxynitrite causes generalised oxidative damage, ***e.g. it.*** reacts with DNA, lipids and proteins [20-25]. Therefore, peroxynitrite is able to disturb many cellular processes, including an enhancement of NF-κB-mediated pro-inflammatory signal transduction pathway [26, 27]. However, it cannot be directly measured *in vivo* due to its nature, formation and decomposition resulting in a short half-life and low steady concentration, but secondary markers can be used, such as the ability of peroxynitrite to nitrosylate tyrosine residues [28]. There are several lines of evidence for the formation of peroxynitrite in biological systems which include the presence of peroxynitrite detoxifying enzymes and the kinetically and thermodynamically favoured reaction between superoxide and nitric oxide. To date there is no data on the presence of peroxynitrite in the intervertebral disc.

Nuclear factor kappaB (NF-κB) plays an important role in the transcriptional regulation of genes involved in inflammation. In resting cells, NF-κB is maintained in an inactive state in the cytoplasm. In response to pro-inflammatory stimuli, translocation of the NF-κB subunits (p65/p50) to the nucleus is rapidly observed at the site where transcription is initiated upon binding of NF-κB to the promoter regions of responsive genes.

The aim of this study is to examine whether peroxynitrite plays a role in the inflammatory cascade, possibly involving NF-κB, which may lead to painful disc

degeneration. In order to mimic the *in vivo* continuous flux of peroxynitrite, the peroxynitrite donor 3-morpholinesydnonimine (SIN-1) was used since it slowly decomposes to release both superoxide and NO which react together to produce peroxynitrite.

METHODS

Cell isolation and culture. Intervertebral disc tissue was obtained from a total of 6 patients (Table 1) and used for either cell culture or formaldehyde fixation. All specimens were collected under informed consent and approved by the institutional review board. Nucleus pulposus cells with their pericellular matrix (*i.e.* chondrons) were isolated by overnight digestion with 0.2% collagenase Ia (Sigma, Switzerland) and 0.3% dispase II (Roche Diagnostics, Switzerland) in PBS (w/v) (37°C, 5 % CO_2). Digested tissue was filtered (70 μm cell strainer, BD Bioscience, Switzerland) and washed three times with DMEM/F12 medium. Cells were expanded up to passage three in monolayer using DMEM/F12 medium, containing 10 % FCS, penicillin (50 units/ml), streptomycin (50 μg/ml), ampicillin (125 ng/ml) (Invitrogen, Switzerland) with medium changes twice a week.

Cell stimulation. Normal medium was replaced by serum-free medium containing 3-morpholinesydnonimine (SIN-1, 1 mM) (Sigma, Switzerland) or 100μM peroxynitrite (Millipore, Temecula, CA, USA) and 2% ITS (Insulin, Transferrin, Selenium) as serum replacement (Sigma, Switzerland) and cells were stimulated for 2, 4 or 6 hours for gene expression, viability and nitrosylation assays.

Analysis of cell viability. Viability of cells treated with SIN-1 or peroxynitrite was assessed by fluorescence staining using calcein-AM (2 μM, Sigma, Switzerland) and

ethidium homodimer-1 (2 μM, Sigma, Switzerland) after 2, 4 and 6 hours. Ethidium homodimer-1 only enters cells with compromised cell membranes and intercalates with nucleic acids producing red fluorescence. Calcein-AM penetrates the membranes of living cells where cytoplasmic esterases cleave the molecule producing green fluorescence. Thus, live cells are depicted green and dead cells red. The percentage viability was calculated manually counting the number of live cells and dividing by the total number of cells visualised per field.

Analysis of nitrotyrosine (immunohistochemistry). Intervertebral disc tissue from two patients undergoing discectomy (Table 1) for degenerative disc disease was analysed to verify the pathological occurrence of nitrosylation *in vivo.* Tissue was fixed, embedded in paraffin, cut (5 μm) and pepsin-mediated antigen retrieval (Pepsin Solution, Lab Vision Corporation, UK) was performed at 37°C for one hour. Sections were then rinsed in PBS and permeabilised with Triton-X (0.2% in PBS). In addition, human NP cells grown on glass slides were stimulated with SIN-1 for 2, 4 or 6 hours, fixed with 4% formaldehyde and permeabilised as described above. Both, cells and tissue sections, were blocked in 1 % BSA (Sigma, Switzerland), 0.2 % dry milk powder and 0.1 % Tween 20 in PBS for one hour, followed by incubation with rabbit polyclonal antibody to nitrotyrosine (Millipore, Temecula, CA, USA, 1:100 dilution) in 1 % BSA and 0.1 % Tween 20 in PBS for one hour. Slides were rinsed in PBS and incubated for 30 minutes with the secondary antibody goat anti rabbit Alexa Fluor 488 (Molecular Probes, Switzerland, 1:400 dilution). They were then rinsed in PBS and nuclear counterstaining was performed using DAPI (Sigma, Switzerland) in PBS (1:1000) for 15 minutes. Slides were again rinsed, mounted with Vectashield (Vector Lab, Inc, CA, USA) and viewed under the fluorescent microscope. As

a positive control, cells were treated with 100 μM peroxynitrite (Millipore, Temecula, CA, USA).

Analysis of intracellular reactive oxygen and nitrogen species. Human NP cells were treated as described for nitrosylation analysis. In brief, cells were incubated in the dark for 30 minutes in PBS containing 10μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Sigma). The ROS generation was detected as a result of the oxidation of DCFH (excitation, 488nm; emission, 515-540nm). Samples were mounted onto slides and imaged under the fluorescence microscope.

Analysis of NF-κB/p65 translocation (immunohistochemistry). Human NP cells were treated as described for nitrosylation analysis. NF-κB/p65 nuclear translocation was analysed using a rabbit polyclonal antibody to NF-κB/p65 (C-20, Santa Cruz Biotech). As a positive control, cells were treated with TNF-α (10 ng/ml) which has been previously shown to induce NF-κB/p65 nuclear translocation.

Gene expression analysis (real-time RT PCR). Cell cultures were stimulated for 2, 4 and 6 hours with SIN-1 (1mM) and total RNA was isolated using a Miniprep Kit (Sigma, Switzerland) according with the manufacturer's instructions. cDNA was synthesised from 10 ng of total RNA using High Capacity cDNA Archive Kit (Applied Biosystems, Switzerland). Human specific probes and primers for IL-1β, IL-6, IL-8, IL-10 and TNF-α (Applied Biosystems, Switzerland) were used for real-time RT-PCR with RG-3000A (Corbett Research). The comparative ct method was used to calculate changes in gene expression, additionally using a standard curve (generated from cDNA from human NP cells) to include PCR efficiency in the calculations. Briefly, expression of genes of interest was normalised versus expression of TATA binding protein (TBP) (ΔCt) and thereafter

versus untreated conditions (= 0 hours) (ΔΔCt). Therefore, expression levels are expressed in arbitrary units, setting the untreated value to 1.

Statistical analysis. Gene expression results were tested for statistical significance applying a t-test with results tested for being different from 1 (= no stimulation) and a significance level of $p < 0.05$.

RESULTS

Nitrosylation in degenerated IVDs.

Nitrosylation occurs in vivo during intervertebral disc degeneration and is primarily located in the NP as shown in Figure 1. Scarce nitrosylation was noted in cells from the AF. Cells from the transition zone, which lies between the AF and NP, did show positive staining for nitrosylation (Data not shown). A 100x magnification picture of a NP cell shows in detail the tyrosine nitration intracellular staining.

Patient number	Sex	Age (years)	Pathology of IVD	Levels
1	Male	25	herniation/sequestered	L4/L5
2	Male	14	traumatic fracture	L4/L5
3	Male	40	traumatic fracture	L5/S1
4	Female	37	Herniation	L5/S1
5	Male	56	Degeneration	L5/S1
6	Female	63	Degeneration	L4/L5

Table 1. Demographical data of the IVD donor and their correspondent pathology.

Nitrosylation in NP cells.

Upon treatment with SIN-1 or authentic peroxynitrite, nitrosylation was detected at 6 hours (Fig. 2a). Our unstimulated cells showed none or little staining. Tyrosine nitration was further analysed for NP cells and stimulated with SIN-1 for all time points (Fig. 3). Nitrosylation was distinctively intracellular. Nevertheless, there was a slight change in the pattern of the staining within the time points. For instance, at 2 and 4 hours, the staining was mainly cytoplasmic, with a higher intensity in the periphery of the nucleus. At 4 hours some cells show nuclear nitrosylation staining whereas at 6 hours most cells give both a cytoplasmic and nuclear staining. Thus, indicating that both cytoplasmic and nuclear proteins get nitrosylated at their tyrosine residues with increasing time. In addition, NP cells stimulated for 14 hours also showed nuclear and cytoplasmic nitrosylation (data not shown).

Intracellular ROS in NP cells.

A strong CMH_2DCFDA staining was observed in NP cells stimulated with either SIN-1 or authentic peroxynitrite. Again, unstimulated cells showed a rather weak fluorescence staining for CMH_2DCFDA (Fig. 2b). The lower cell number of the pictures for peroxynitrite and SIN-1 is not due to decreased cell viability but was chosen on purpose by plating lower amounts of cells so as to reduce the background of the CMH_2DCFDA staining.

Cell viability of NP cells.

There was no effect observed on the cell viability (>98%) upon stimulation with either, SIN-1 or peroxynitrite for 6 hours (Fig. 2c). NP cells remained viable at 96% for at least 14 hours of stimulation with SIN-1 (Data not shown).

NF-κB subunit p65 translocation into the nucleus.

As shown in Figure 3b, NF-κB/p65 was present in the nucleus at 2, 4 and 6 hours, becoming more abundant with increasing time. Lime-green arrows indicate a representative example of a cytoplasmic staining of NF-κB/p65. Nuclear translocation of NF-κB/p65 becomes more abundant with increasing time. Blue arrows indicate a typical nuclear staining for NF-κB/p65. A positive control was run in parallel with NP cells stimulated with TNF-α (data not shown). Furthermore, NF-κB/p65 still remained in the nucleus after 14 hours of stimulation with SIN-1 (data not shown).

Gene expression on SIN-1 treatment.

Human NP cells were stimulated for 2, 4 and 6 hours with 1mM SIN-1 and the expression of IL-1β, IL-6, IL-8, IL-10 and TNF-α were quantified using real time RT-PCR. Interestingly the gene expression pattern varied among the cytokines tested. Gene expression for IL-1β gradually increased with time, registering a statistically significant 3.5 and 4.5 fold increase at 4 and 6 hours, respectively, of treatment with SIN-1. The opposite pattern was quantified for IL-6 with a 4 fold increase peak as early as 2 hours of stimulation, but returning immediately to basal levels at 4 hours. The pattern of IL-8 was similar to IL-6 reaching a 9.75 fold increase at 2 hours stimulation. Nevertheless, cytokine

levels remained at a constant 3 fold increase thereafter. Moreover, TNF-α and IL-10 levels remained constant and unchanged throughout the treatment for the time points tested.

DISCUSSION

Oxidative stress has long been related to IVD degeneration and other inflammatory joint diseases [1, 15, 25, 29]. Whilst most studies focus on the occurrence and effects of reactive oxygen species (ROS), reactive nitrogen species (RNS) have been shown to be involved in degenerative diseases, although mechanisms and effects are far less understood to date, especially with regards to IVD degeneration. So far, research tackling discogenic degeneration has mostly dealt with NO and inducible NO synthase (iNOS) [5, 6, 9]; scarce or no information is available on peroxynitrite, despite being an even more reactive, damaging and toxic species [17]. Peroxynitrite is one of the major damaging oxidants produced in mammals during aging, inflammation and neurodegenerative disorders among other pathologies. However, its role during IVD degeneration and its putative influence on pain initiation is yet unknown. Several of the biological effects of peroxynitrite which could be related and involved in disc degeneration include inhibition or depletion of antioxidant enzymes, activation of specific enzymes such as matrix metalloproteinases [30, 31], impairment of enzyme cofactors (BH_4) [32], DNA injury [33]and mitochondrial dysfunction [34]. Moreover, peroxynitrite has been shown to also modify receptors, causing downstream receptor coupling and signalling with the activation of MAPK pathway, PARP, NF-κB, phosphoinositide 3-kinase/Akt, PKCε, cyclo-oxygenase and nerve growth factor [35-43].

Based on this knowledge, we assumed that peroxynitrate may play an important role during IVD degeneration and may, additionally, induce synthesis of pro-inflammatory cytokines shown to increase in painful disc degeneration. It is thought that pro-inflammatory cytokines can diffuse through the disc via clefts and tears and may therefore irritate nerve roots and nerve endings in the epidural space or the outermost part of the annulus fibrosus.

In order to test this hypothesis, native degenerated IVD tissue was analysed and found to be positive for nitrosylation. However, we were not able to analyse nitrosylation in healthy IVD tissue in comparison, so we can not conclude that nitrosylation is a process that is specific to degenerated tissue. Nevertheless, NP chondron-cells isolated from those same discs were only positive for nitrosylation and intracellular ROS when stimulated with peroxynitrite or SIN-1.

In a second step, we performed experiments on cells isolated from human IVD tissue. To minimise cellular and phenotypical changes induced by the isolation and expansion, cells were isolated with their pericellular matrix (chondrons) and not expanded further than passage 3. In this cell culture model, we were able to show that SIN-1, a peroxynitrite donor, and authentic peroxynitrite (data not shown) induced gene expression of IL-1β, IL-6 and IL-8 with distinct patterns for all genes. Whilst IL-1β increased time-dependently, IL-6 and IL-8 both had a strong initial peak at 2 hours followed by a decrease in the later time points tested. Note that gene expression for IL-6 returned to basal levels whereas IL-8 remained upregulated at 4 and 6 hours. In contrast, no changes in gene expression for IL-10 and TNF-α could be found; thus highlighting the importance of IL-1β, IL-6 and IL-8 during nitrosative stress. Oscillatory wave curves were detected for these three NF-κB-dependent cytokines, IL-1β, IL-6 and IL-8, indicating that even though NF-κB might be a shared

transcription factor for these genes, the recruitment onto the promoter, its accessibility and the involvement of various co-regulators might be rather different, leading to time differences. In addition, these cytokines have been related to IVD degeneration and even herniation. These findings are in line with a study performed by Le Maitre et al. [44, 45], showing that IL-1β but not TNF-α can be detected in human degenerated IVDs.

In a third step, we aimed to identify the pathway underlying the observed changes in gene expression. As nuclear factor kappaB (NF-κB) plays an important role in the transcriptional regulation of genes involved in inflammation, we hypothesised its involvement in this pro-inflammatory cascade. We were able to show NF-κB/p65 nuclear translocation due to stimulation with SIN-1, suggesting the involvement of NF-κB during oxidative stress as simulated in this cell culture model. Interestingly, NF-κB/p65 nuclear localisation was not transient, but was observed to increase throughout the time points tested and was still present after 14 hours (data not shown). Therefore, peroxynitrite stimulation does not seem to act on the so-called canonical or classical pathway. Instead an alternative mechanism of NF-κB activation seems to be taking place under prolonged exposure of NP cells to peroxynitrite. This pathway would consist of the constitutive activation of NF-κB through the tyrosine nitration of I-κB [46, 47] which has been already described in detail in other tissues/cells such as in muscle myoblasts [27]. Long term exposure to peroxynitrite leads to the nitration of I-κB which inhibits its degradation by the ubiquitin and proteasome pathway and thereby activates continuous and non-transiently NF-κB [48] [49]. The continuous and long term activation of NF-κB may exacerbate the inflammatory responses mediated by this transcription factor.

Our results suggest that oxidative/nitrosative stress caused by peroxynitrite might play an important role in the development and persistence of discogenic inflammation as it activates an NF-κB dependent signal transduction pathway and promotes enhanced gene expression of NF-κB dependent cytokines [50], related to IVD degeneration. Significantly, this effect was not only transient, but was persistent, therefore highlighting its potential importance in prolonged inflammatory states. Antioxidant treatment or tyrosine nitration inhibition might become a treatment option for disc degeneration and related discogenic pain.

In conclusion, our study suggests that peroxynitrite may play a role in the inflammatory cascade occurring during disc degeneration and could eventually lead to discogenic back-pain.

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LEGENDS

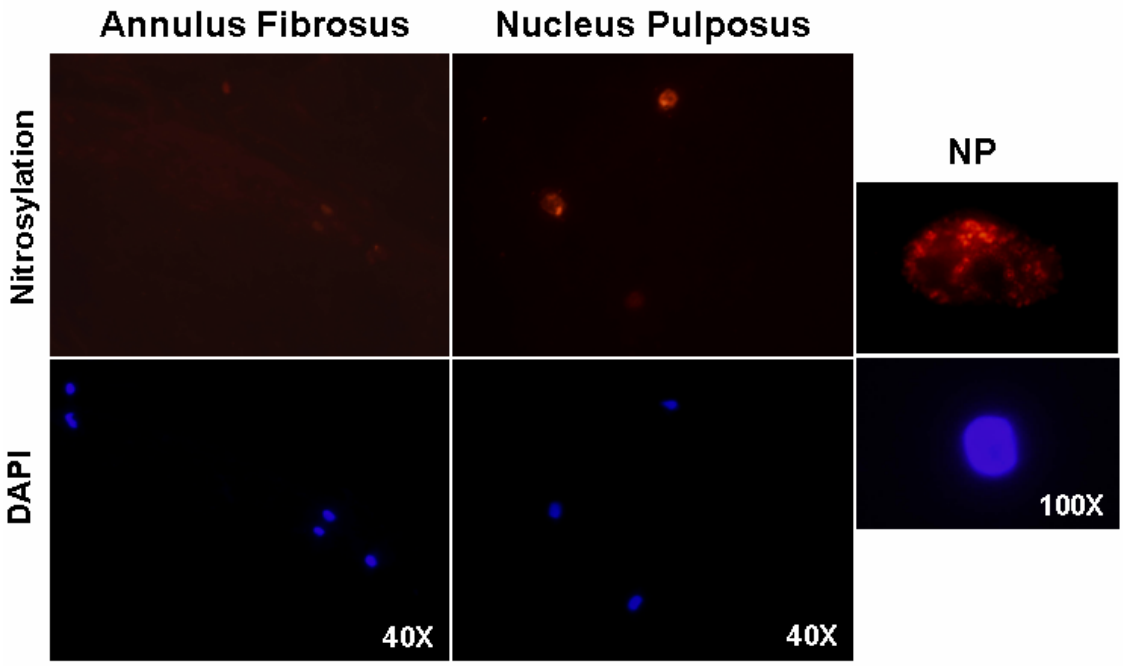
Figure 1. Nitrosylation in the human native nucleus pulposus tissue and annulus fibrosus. Immunolocalisation of nitrosylation in native human AF *(first column)* and NP tissue *(second column)*. Pictures in the third row correspond to a picture in a higher magnification (100x) of NP cells in the native tissue (blue = nuclear stain with DAPI, red = nitrosylation).

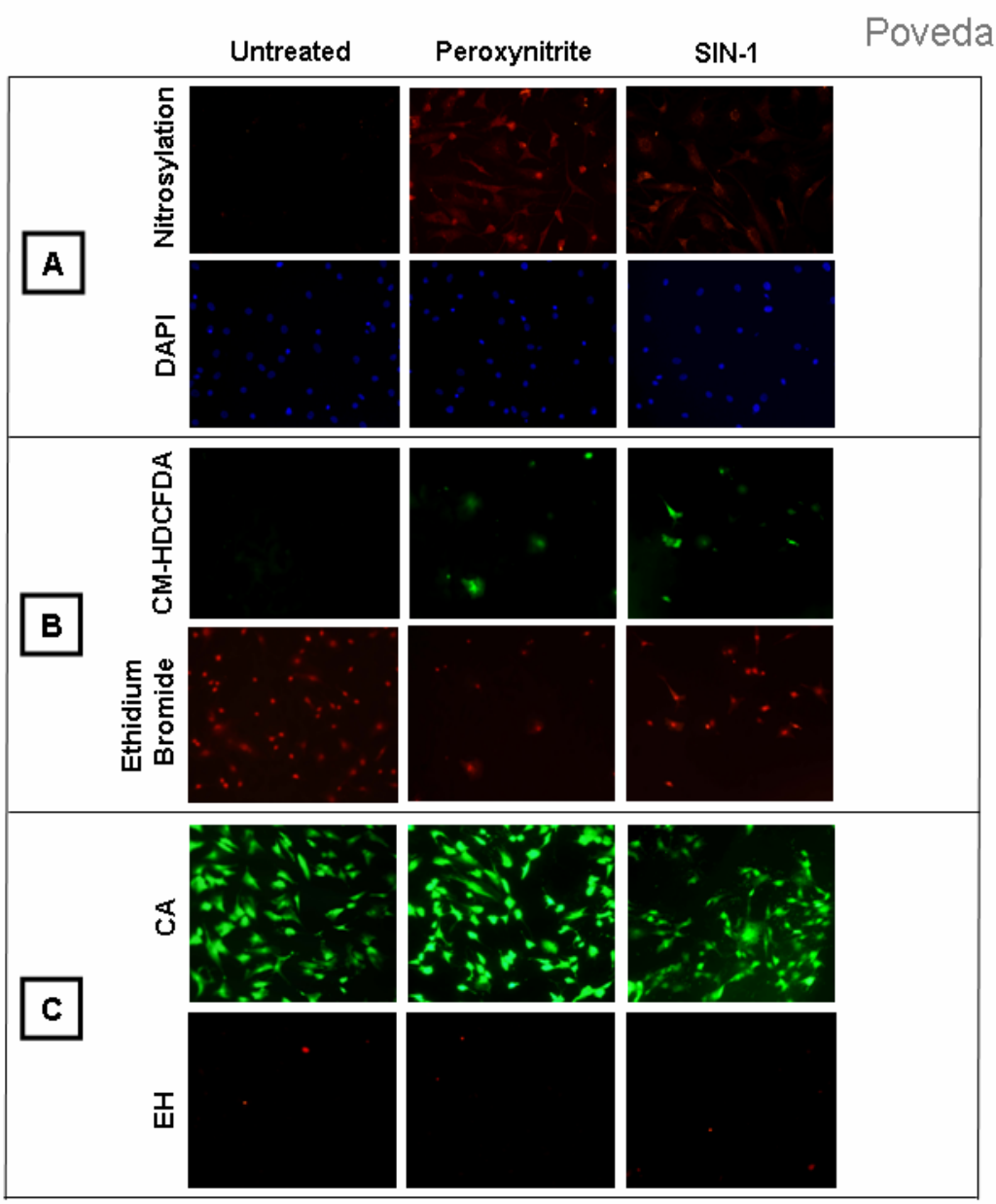
Figure 2. Reactive oxygen/nitrogen species in human nucleus pulposus cells cultured with either peroxynitrite or SIN-1 for 6 hours. Immunolocalisation of nitrosylation *(Panel A)*, or intracellular reactive oxygen/nitrogen species *(Panel B)* in human NP cells stimulated for 6 hours with either 100μM peroxynitrite or 1mM SIN-1. The cells were counterstained in *Panel A* and *Panel B* with DAPI and ethidium bromide, respectively. Cell viability *(Panel C)* was performed using calcein-AM (green = viable cells) and ethidium homodimer (red = dead cells).

Figure 3. Effects of SIN-1 in human nucleus pulposus cells. Immunolocalisation of nitrosylation *(Panel A)* or NF-κB/p65 nuclear translocation *(Panel B)* in NP cells cultured for 0, 2, 4 and 6 hours with 1mM SIN-1. Nuclear counterstain was obtained with DAPI. The lime-green arrow corresponds to a typical cytoplasmic staining whereas the blue arrow indicates an example for a nuclear staining.

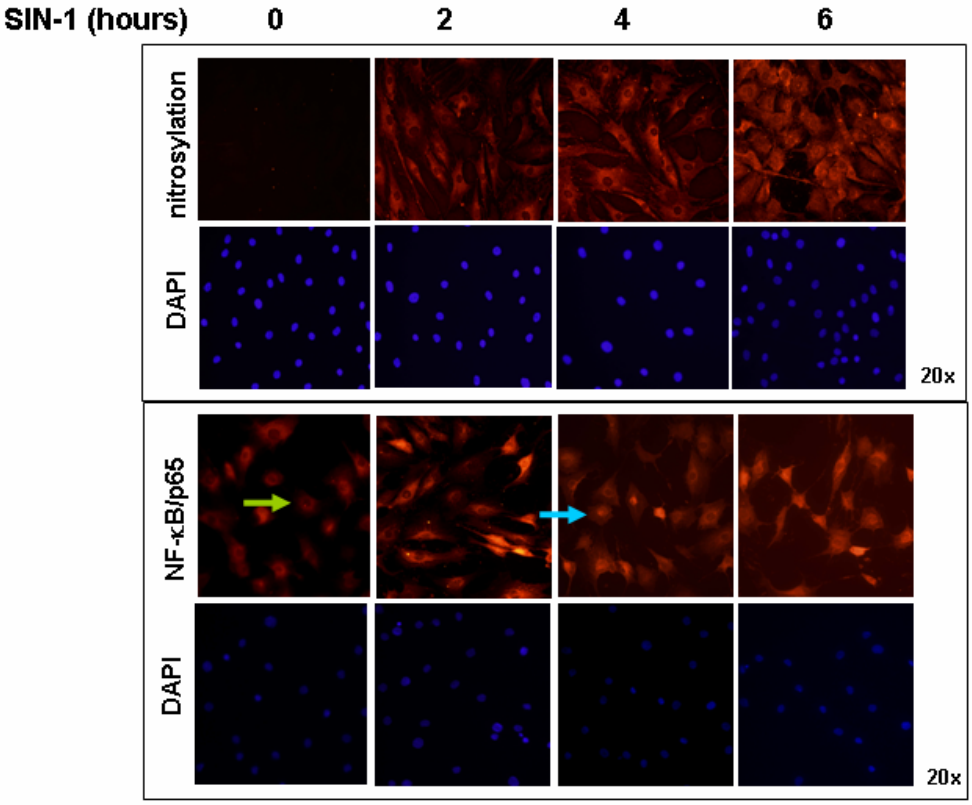
Figure 4. Pro-inflammatory cytokine profile of human nucleus pulposus cells stimulated with SIN-1. All experiments were performed under normal culturing conditions. The cytokine profile for IL-1β, IL-6, IL-8, IL-10 and TNF-α was analysed using quantitative real-time RT-PCR. On the *y* and *x* axis, the fold increase and the time point in hours is represented, respectively. Expression levels were expressed arbitrary units, setting time 0 hours value as 1. The values were normalised relative to the reference gene TBP to compensate for differences in cDNA loading. This figure represents a mean of at least five biologically independent samples. * corresponds to $p < 0.05$, all *p* values were relative to time 0 hours.

Poveda et al. Figure 1

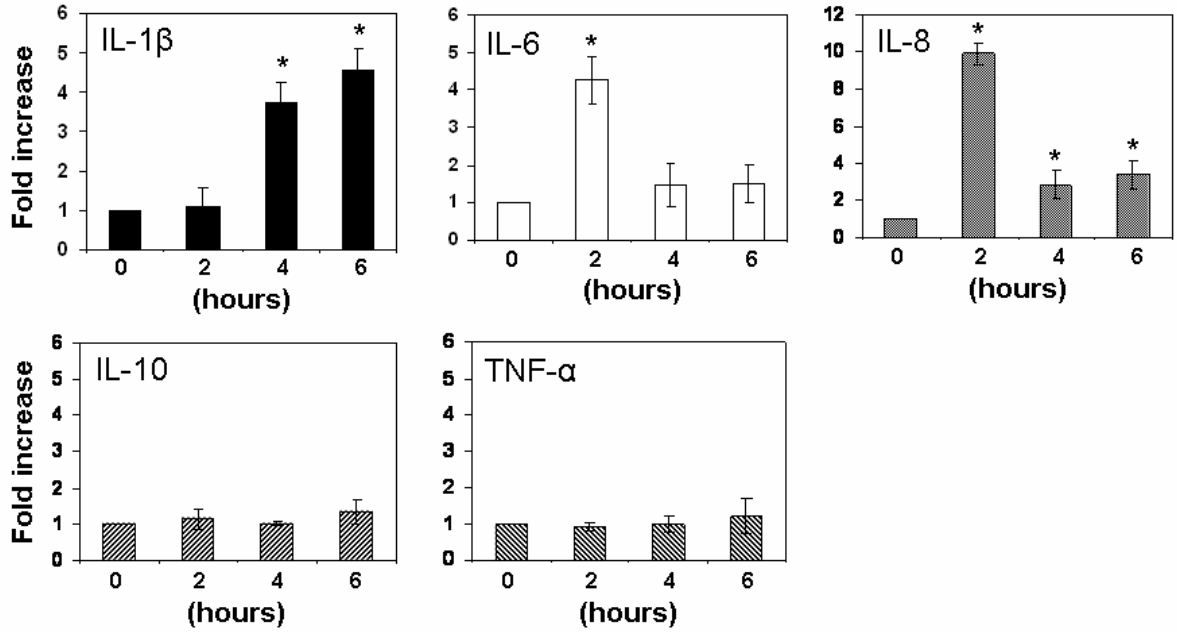



Poveda et al. Figure 2
Untreated
Peroxynitrite
SIN-1
A
Nitrosylation
DAPI
B
CM-HDCFDA
Ethidium Bromide
C
CA
EH

Poveda et al. Figure 3



Poveda et al. Figure 4



6. Unpublished results

6.1. Environmental condition-dependent accumulation of N-(carboxymethyl) lysine (CML) and expression of RAGE (receptor for glycation end products) in a chondron-based pellet culture system of bovine nucleus pulposus cells.

6.1.1. Introduction and aim

Recently, Nerlich *et al.* investigated occurrence of the post-translational protein modification N-(carboxymethyl) lysine (CML) in autopsy specimens of lumbar intervertebral discs from individuals of different age groups and stages of degeneration [25]. CML has been shown to emerge from so-called advanced glycation end products (AGE) after a series of oxidation and rearrangement steps [325-327]. Overall, CML may serve as a biomarker of general oxidative stress [328]. Immunolocalisation of CML in the intervertebral disc revealed age-dependent CML deposition in areas of macroscopic and histological degeneration of the IVD matrix [25]. This agrees with age-dependent accumulation of CML in other tissues consisting of long-lasting matrix proteins [329-331]. Accumulation of CML and other AGE is recognised by the multi-ligand receptor RAGE (receptor for advanced glycation end products), a member of the immunoglobulin superfamily [113, 114]. Ligand-binding of RAGE has been shown to activate key signalling pathways such as NF-κB, potentially resulting in a pro-inflammatory signalling cascade involving pro-inflammatory cytokines [113].

We hypothesise that accumulation of CML in the ageing intervertebral disc matrix might initiate a signalling cascade that finally results in the expression of pro-inflammatory

cytokines, which might be responsible for the induction of discogenic low back-pain. Analysis of the environmental conditions leading to CML accumulation *in vivo* is hampered by the limited possibilities to explore such conditions inside the IVD *in vivo*. Investigations on the signalling cascade within the disc therefore require the application of cell culture models. Bovine IVDs as a cell source are advantageous because i) they are phenotypically similar to human nucleus pulposus cells, ii) the IVDs are easily accessible, and iii) the heterogeneity in the cell population is much less a problem than in normal adult discs which can exhibit a larger variation in degenerative changes [148, 332, 333]. In an attempt to avoid the introduction of foreign matter and to preserve the original phenotype/environment of disc cells, we made use of the natural self-organising, pellet-forming capability of disc cells. A carrier-free 3-dimensional culture system was used which allowed disc cell self-rearrangement, production of ECM and above all to preserve the original phenotype/environment of disc cells by isolating disc cells with their PCM (chondrons).

In using a bovine chondron-based cell culture model, we investigated the accumulation of CML and expression of RAGE after subjecting the pellet cultures to various environmental conditions which may resemble the aggravated environment that disc cells might encounter during the process of degeneration.

6.1.2. Results

Isolation of bovine nucleus pulposus-derived chondrons. First, we determined the feasibility of isolating NP cells with their pericellular matrix (PCM). According to the terminology in cartilage, cells plus PCM are termed chondrons. All discs used in this study

were morphologically very similar, with a white NP that was clearly distinguishable from the AF. Morphology of the discs was comparable to Grade II discs according to Thompson et al. [31].

Digestion of NP tissue with 1 % collagenase-only or with a mixture of 0.3 % dispase/0.2 % collagenase (adapted from [334]) led either to chondrocyte-like single cell preparations or to preparations containing mostly chondrons. The presence of the pericellular matrix was confirmed by immunolocalisation of collagen type VI, which has been shown to be preferentially localised in the pericellular matrix of disc cells and was mostly absent in the 1 % collagenase-only digested cultures [335] (Fig. 5A and B). This suggests the presence of the PCM in the chondron suspension used for subsequent pellet formation. Immunodetection of collagen type II, a marker for the differentiated disc cell phenotype, revealed that the nucleus pulposus-derived cell suspension versus the chondron suspension had a slightly higher expression of collagen type II (Fig. 5C – G). Note the accumulation of collagen type II at certain foci around the cell (Fig. 5G). Viability of the cells was generally higher than 98% as determined by Trypan blue exclusion.

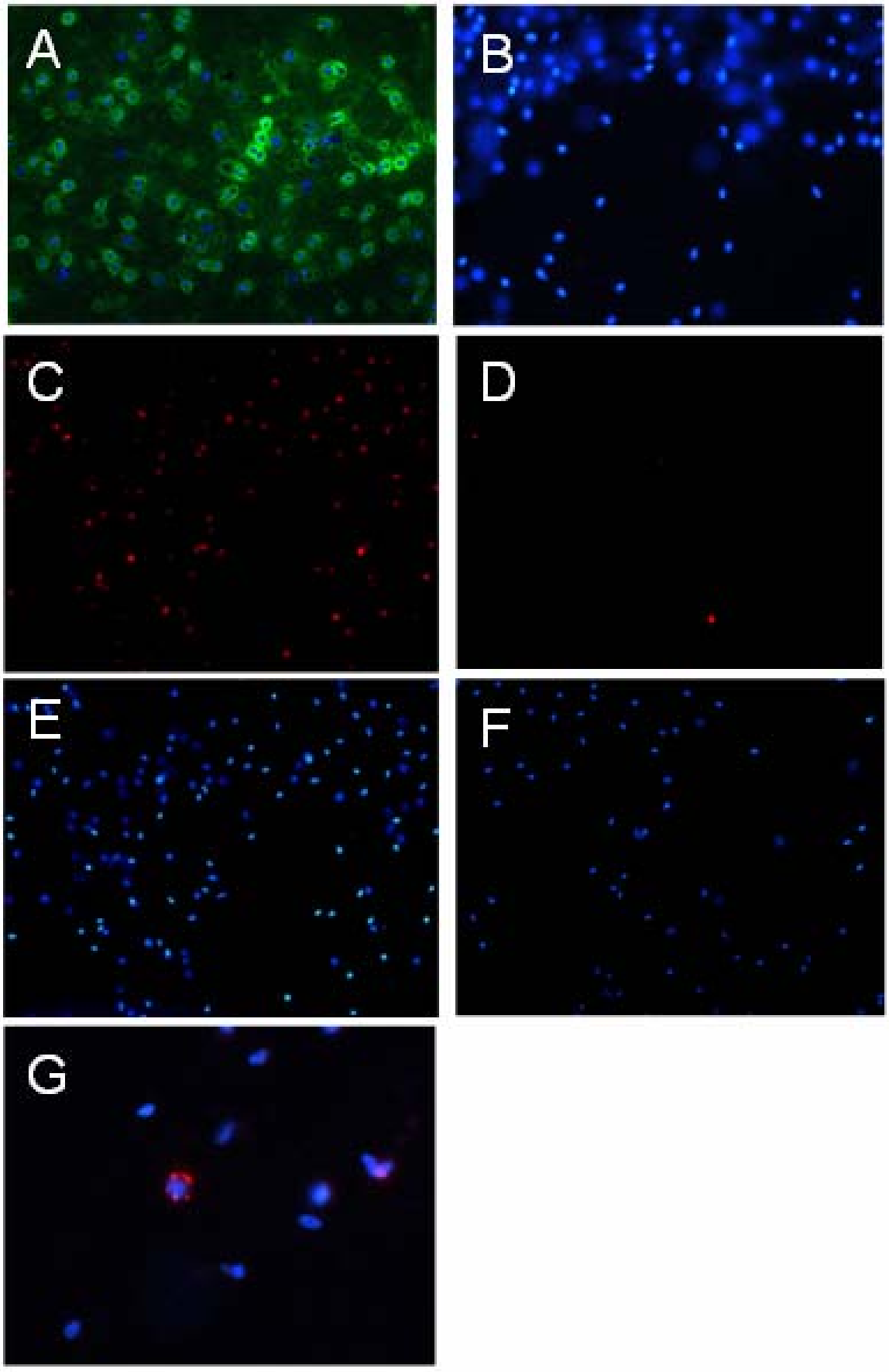

A
B
C
D
E
F
G

Figure 5. Immunolocalization of collagen type VI and II in cell and chondron suspensions of nucleus pulposus tissue. Immunofluorescence staining to detect collagen type VI chondron-suspension (A) and cell-suspension (B). Collagen type VI is detected as a halo surrounding the cell (green), cell nuclei were counterstained with DAPI (blue). C and D: cellular distribution of collagen type II in chondron (C) or cell (D) suspensions. E and F: corresponding nuclear stain with DAPI. G: foci of collagen type II accumulation (red = collagen type II, blue = nuclear staining DAPI). Original magnification: A – D: 20 x, E: 40 x

Routine histology revealed notochordal-like cell clusters in cell and chondron suspensions (Figure 6).

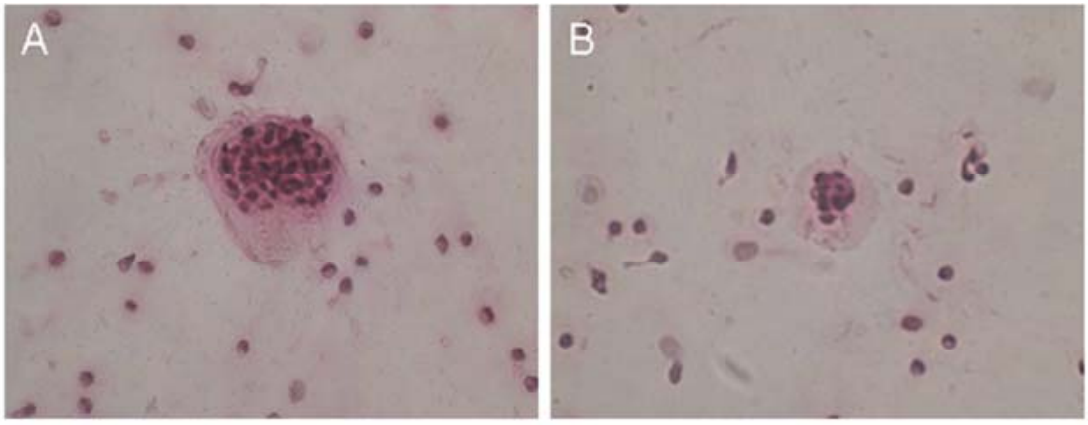


Figure 6. Notochordal-like cell clusters in NP-derived cell or chondron suspensions. Haematoxylin and eosin staining revealed notochordal-like cell cluster in cell (A) and chondron (B) suspensions. Original magnification: 40 x

Pellet formation from NP-derived chondrons. Within seven days of the onset of the experiment the pellets were readily formed. At four weeks, the pellets were removed from the medium and processed for routine histology. Haematoxylin and eosin staining revealed the spherical shape of the pellets with the cells embedded in considerable amounts of extracellular matrix (Fig. 7). Proteoglycan, an important component of the NP extracellular matrix was found present in the pellet matrix as detected by histology (Fig.8 A). Several cells were surrounded by lacunae, similarly to disc cells in the native NP (Fig. 8A, arrows).

The pellet was surrounded by a thin layer of densely packed cells not separated by extracellular matrix. The presence of proteoglycans was further confirmed by a colorimetric assay for sulfated glycosaminoglycans (GAG) (Fig. 8B). The GAG content remained constant up to four weeks after incubation (Fig. 8B). Longer incubation led to an increase of the GAG content. Immunodetection of the proliferation marker Ki-67 was negative, suggesting that the observed increase of proteoglycans is not due to cell proliferation (data not shown). The GAG content of the pellets was approx. 20 times lower than that found in native bovine NP tissue (7.9 µg GAG / mg tissue) and 10 times lower when compared to bovine AF tissue (4.7 µg GAG / mg tissue).

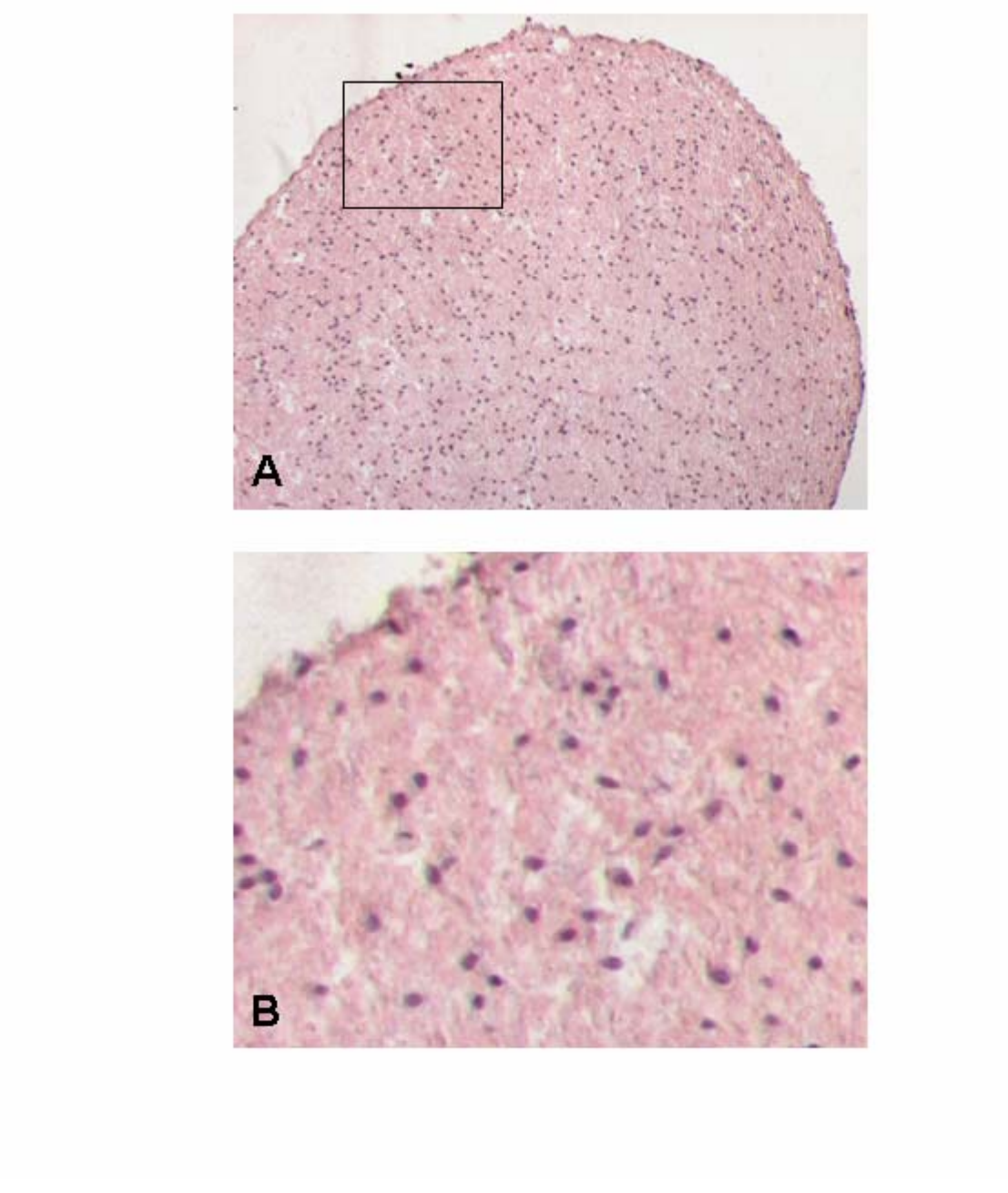


Figure 7. Haematoxylin and eosin staining of a chondrons-based pellet.
A) Note the round shaped morphology of the pellet culture system. B) Enlargement of the area indicated in (A).

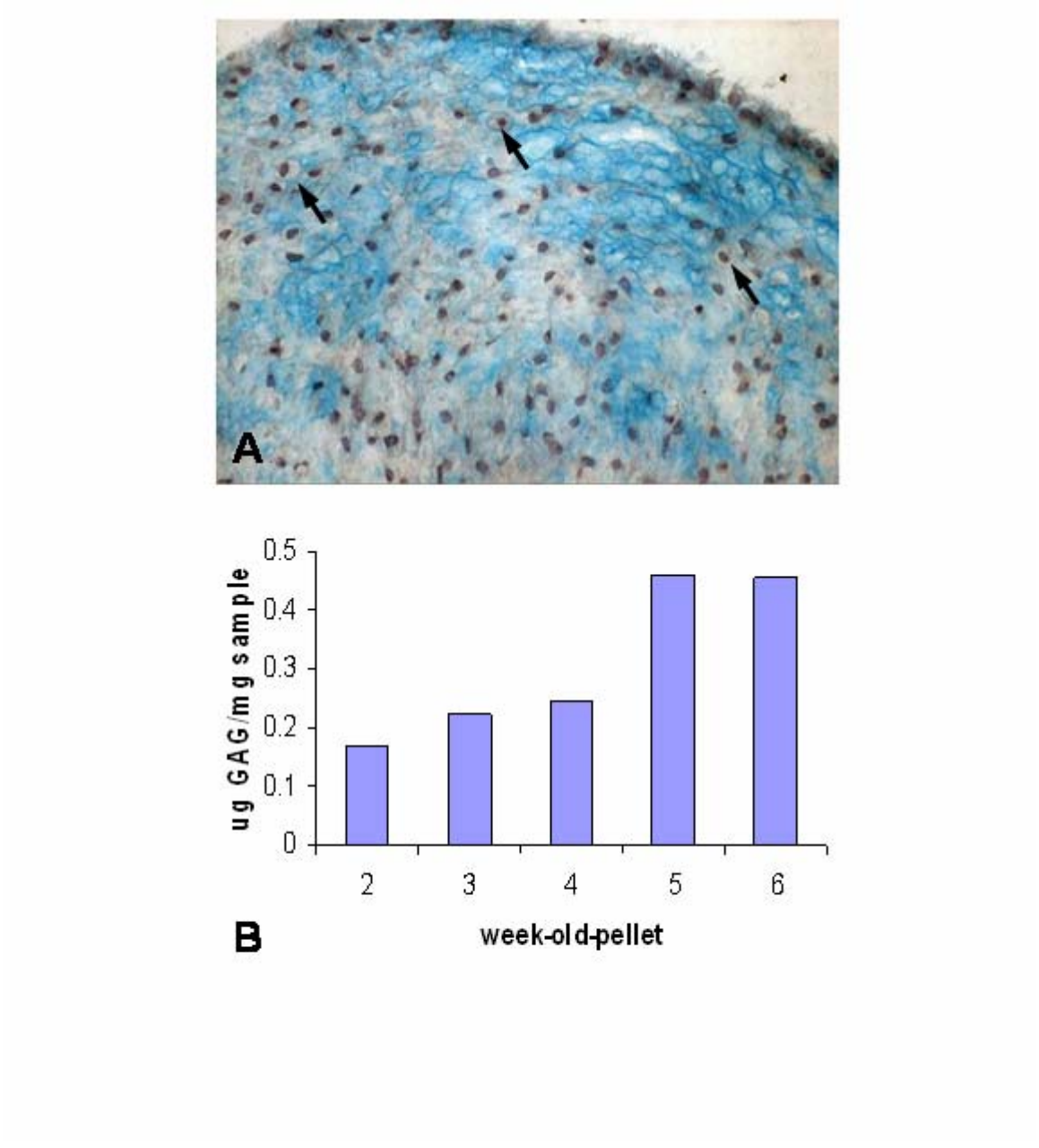


Figure 8. Presence of proteoglycan in the chondron-based pellet. A) Alcian blue staining of a chondron-based pellet. Cells were counterstained with Mayer's hemotoxylin, proteoglycans appear blue. B) Proteoglycan content of the chondrons-based pellet culture at indicated time points. Expressed as µg sulphated glycosaminoglycans per mg wet weight of the pellet tissue. The experiment was performed on two independent samples (measurements in duplicates) and averages are presented.

The presence of collagens was determined after four weeks of incubated pellets by Van Gieson staining for collagen type I or by immunohistochemistry for collagen type II.

Due to the strong interactions with acidic dyes, type I collagens can be demonstrated more selectively with compound solutions of acidic dyes like van Gieson [336]. Staining of native bovine disc tissue from the transition zone and the nucleus pulposus revealed intense red areas in the transition zone that were absent from nucleus tissue (Fig. 9AB). In the pellet we observed the intense red staining exclusively in the outermost cell layer (Fig. 9C), suggesting the absence of fibrous collagens inside the pellet. Immunohistochemistry for collagen type II in native bovine disc tissue revealed most intense staining in the PCM surrounding the disc cells (Fig. 9DE). In the pellet, collagen type II was also detected, but the localisation was more outspread (Fig. 9F).

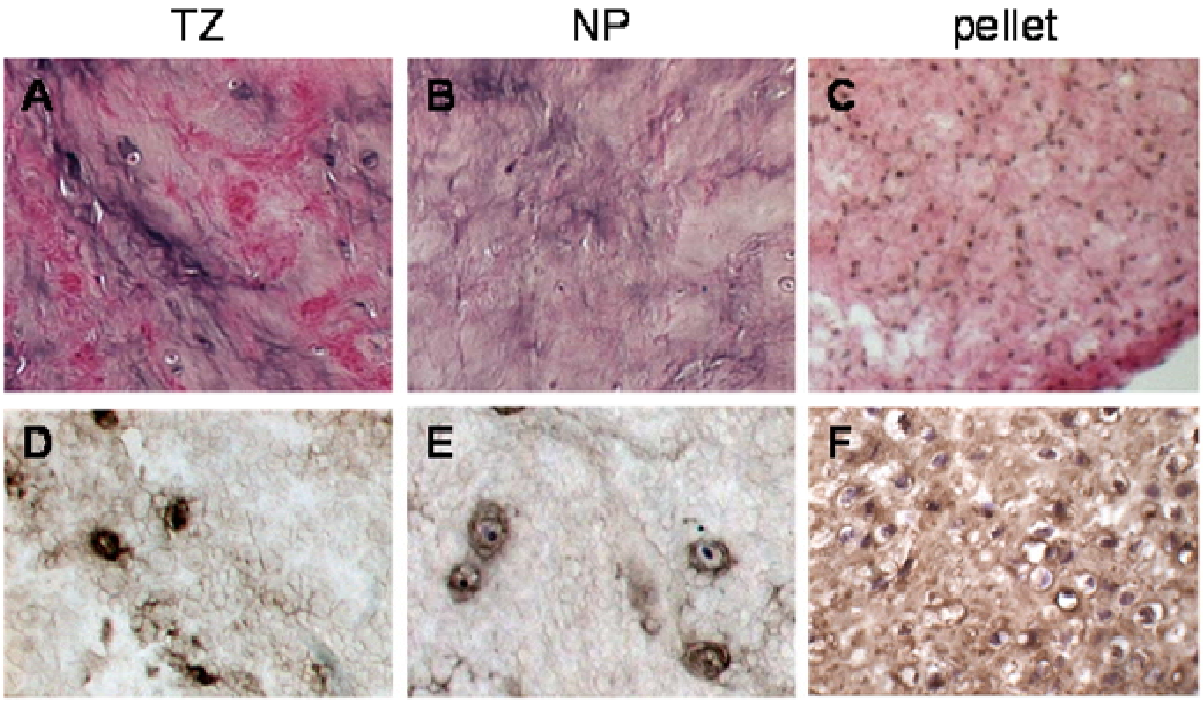


Figure 9. Van Gieson and collagen type II-specific immunostaining of the TZ and NP of the IVD and of the pellet. Van Gieson staining of the transition zone (TZ) of the bovine IVD (A), nucleus pulposus (NP) (B), and pellet (C). Collagen type II DAB immunostaining of the TZ (D), NP (E), and pellet (F). Positive staining for collagen type II appears brown; nuclei are counterstained with Mayer's hematoxylin.

Accumulation of CML-modifications after exposure to various environmental conditions.

CML accumulation was analysed in chondron pellet cultures exposed to environmental conditions. Incubation of the pellets in cultivation medium containing the more reactive reducing sugar ribose in replacement of glucose to accelerate non-enzymatic glycation served as positive control. After 4 weeks of incubation significant accumulation of CML was detected (Fig. 10A). The most intense staining was seen in the vicinity of cells, especially cell clusters that were present in the pellet. However, the extracellular matrix was also often positive. Regions of intense staining were interspersed with cells that did not reveal any positive staining. Considerable weaker staining was found in pellets incubated in standard culture medium, *e.g.* DMEM/F12 containing 200 mM glucose. Again, intense staining was seen in the vicinity of cells and cell clusters with fainter staining intercellularily (Fig. 10B). Hypoxic conditions resulted in a slightly lower intensity as seen with the standard medium. However, more cells were found without CML accumulation in their immediate vicinity (Fig. 10C). Lowering the pH of the medium to pH 6.7 led to a notable reduction of the CML accumulation compared to the standard culture medium. The surrounding of the majority of the cells was negative or only very faintly CML positive (Fig. 10D). Without glucose in the medium, only a small portion of the cells were positive for CML in their vicinity. In intercellular regions only isolated patches were positive for CML accumulation. Surprisingly, we noticed the outermost cell layer to be positive (Fig. 10E). Pellets formed during these conditions typically displayed an irregular shape compared to pellets formed under standard conditions.

Expression of RAGE after exposure to various stress conditions. Since CML accumulates inside the pellet cultures under various conditions we examined the presence of the AGE receptor RAGE. Treatment of the cultures with ribose instead of glucose resulted in almost all cells being positive for RAGE with no staining of the intercellular matrix (Fig. 10G). Pellets incubated with standard medium revealed a very similar pattern compared to ribose-treated cultures, with almost all cells being positive. However, the signal was notably weaker than in the positive control (Fig. 10H). Staining was weaker under hypoxic conditions with many cells being negative for RAGE (Fig. 10I). Lowering the pH resulted in weaker staining compared to the standard medium. However, the staining was more intense and more cells were positive compared to the pellets cultivated under hypoxic conditions with the most intensely stained cells located towards the outside of the pellet (Fig. 10J). Absence of glucose from the medium resulted in disappearance of almost any RAGE-positive cell from the pellet. Very few weakly stained cells that were distributed randomly among the pellet matrix were identified(Fig. 10K).

In conclusion, conditions that led to a strong increase in CML accumulation resulted also in a robust presence of RAGE. However, hypoxia appears to result in a notable decrease in RAGE expression, whereas a lowered pH of the medium did not have any noteworthy effect on RAGE expression. The lack of glucose abolished RAGE presence almost completely (summarised in table 1).

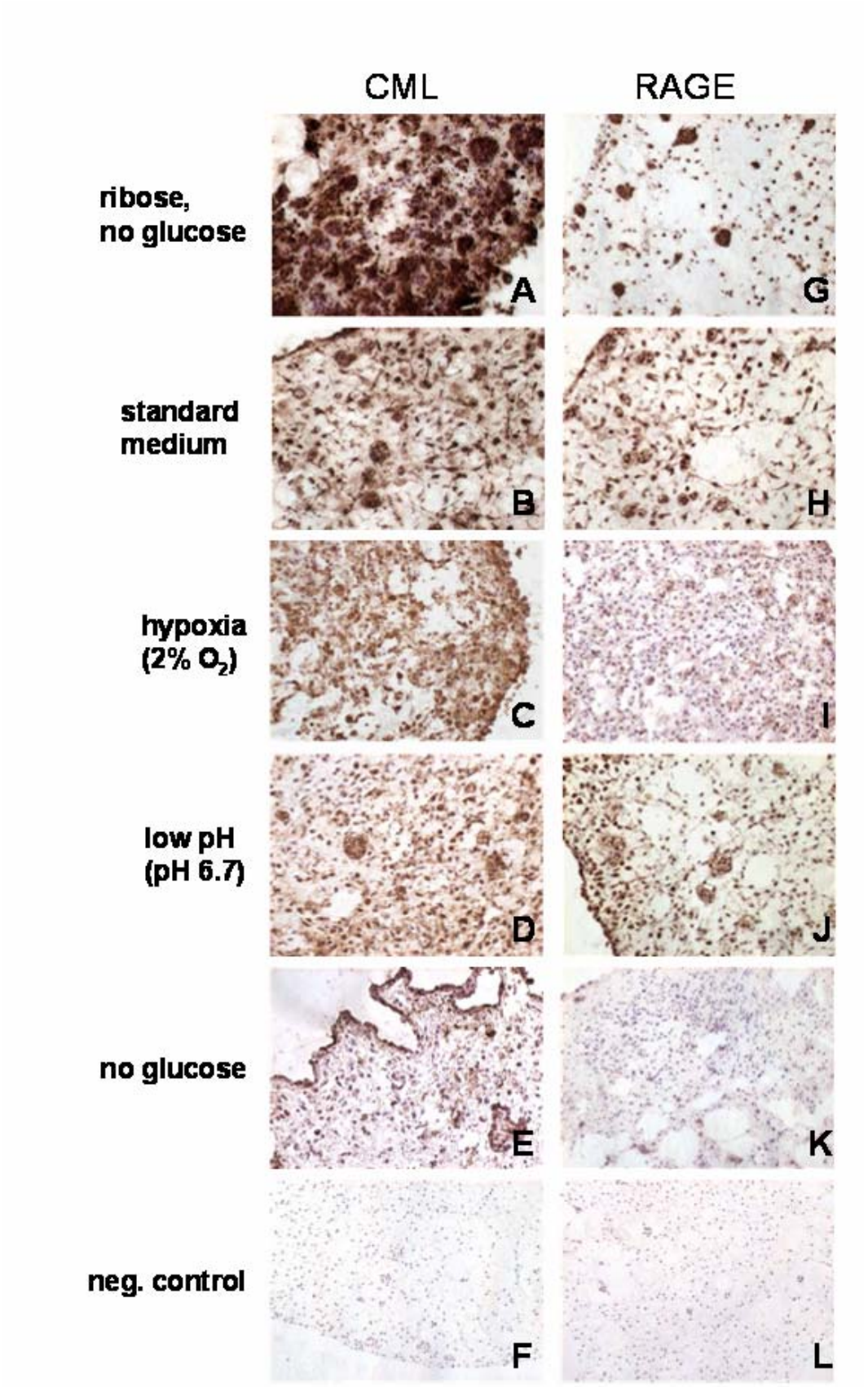

CML
RAGE
ribose, no glucose
standard medium
hypoxia (2% O_2)
low pH (pH 6.7)
no glucose
neg. control
A
B
C
D
E
F
G
H
I
J
K
L

Figure 10. DAB immunostaning for CML and RAGE of chondron-pellets incubated under various stress conditions. Panels A-F and G-L correspond to immunostaining to detect CML and RAGE, respectively. Cultivation conditions of the respective pellets are mentioned on the left (A and G: ribose-containing medium; B and H: standard medium; C and I: hypoxic condition (2% O2; D and J: low pH (pH 6.7); E and K: no glucose medium; F and L: negative controls without primary antibody).

Cell viability after exposure to various cultivation conditions. Assessment of cell viability in the pellets incubated under the various cultivation conditions using a TUNEL assay revealed significant amounts of dead cells in the pellets (Table 1). The highest proportion of dead cells was found when glucose in the cultivation medium was replaced by ribose. Under this condition most of the cells were TUNEL-positive (> 90 %). The presence of 200 mM glucose or the absence of glucose from the medium resulted in a very similar proportion of dead cells in the pellets (54 vs. 57 %). Cultivation under hypoxic or low pH conditions results in a similar proportion of dead cells (48 vs. 49 %), slightly lower compared to cultivation under standard conditions. These results reveal that cultivation of disc cells in pellet cultures for 4 weeks results in a substantial reduction of cell viability. Pellet cultured for 2 weeks revealed a cell viability >90% (Data not shown).

	Incubation conditions				
	ribose	standard	hypoxia	low pH	no glucose
CML	+++++	+++	++	++	+
RAGE	+++++	+++	++	+++	0
pellet morphology	round	round	irregular	Round	Irregular
cell death (%)[a]	92	57	54	48	49

[a] average percentage of two independent experiments

Table 1: CML accumulation, RAGE expression and pellet morphology of the pellets cultivated under various conditions (+++++ = maximal observed intensity, 0 = not detectable).

6.1.3. Materials and methods

Cell Isolation. Bovine caudal spines were obtained from the local abattoir (age approx. 2 years) and processed within 3 hours of slaughter. The discs were dissected and the nucleus tissue carefully separated from the annulus. Nucleus cells with their pericellular matrix were isolated by collagenase/dispase digestion (0.3 % dispase and 0.2 % collagenase w/v in OK medium). Digestion was carried out overnight at 37° C (100 % humidity, air, 5 % CO_2). Digested tissue and medium were filtered with a Falcon 70 μm cell strainer to remove undigested fragments, washed three times with OK medium and resuspended in OK medium. Viable cells, as determined by Trypan blue exclusion, were counted and diluted to 1 x 10^5 cells per ml in DMEM. 5 x 10^5 cells were transferred to a 15 ml polypropylene conical tube (Falcon) and centrifuged at 200 x g to form aggregates. The cultures were incubated at 37° C (100 % humidity, air, 5 % CO_2) either in OK medium (pH 7.2), acidic OK medium (pH 6.7), OK medium in a hypoxic chamber (2% oxygen), DMEM without glucose or DMEM without glucose complemented with 30mM D-ribose. The medium was changed after five days and every three days thereafter.

Materials. OK medium: 50 % DMEM and 50 % F12, 10 % fetal calf serum, 2 mM glutamine, 1 % Antibiotic/antimycotic (penicillin, streptomycin, amphotericin B (Life Technologies)). DMEM without Glucose containing 10% fetal calf serum, 2 mM glutamine and 1 % Antibiotic/antimycotic.

Collagenase (Sigma), dispase II (Roche Applied Science) and D-ribose (Sigma). Ca/Mg-free PBS (PAN Biotech GmbH).

Antibodies: the following antibodies were used: rabbit polyclonal antibody to collagen type II (Acris Antibodies GmbH), rabbit polyclonal antibody to collagen type VI (Acris Antibodies GmbH), rabbit polyclonal antibody to N-(carboxymethyl)-lysine (CML) (E. Schleicher, Tübingen) and mouse monoclonal antibody to RAGE (Chemicon International). Secondary antibodies used in immunohistochemistry: goat anti rabbit Alexa Fluor 488 antibody (Molecular Probes) and biotinylated anti rabbit antibody (Vector Laboratories, Inc).

Histology. Tissue samples were fixed in 3.7% formaldehyde in PBS for 1 day and embedded in paraffin. The tissue sections were heated for 1 hour (60°C), deparaffinized in xylol and rehydrated through graded ethanol. In addition, samples were taken from the cell suspensions yielded after digestion and dried on a Superfrost Ultra Plus® (Menzel GmbH) slide and fixed with 3.7% formaldehyde.

Routine stainings with Alcian Blue (pH 2.5), Haematoxylin & Eosin and Van Gieson were performed on 5 µm tissue sections (according to the manufacturers' recommendations).

Immunofluorescence staining: Pepsin antigen retrieval (Pepsin Solution, Lab Vision Corporation) was performed on 5 µm tissue sections at 37°C for one hour and then rinsed in PBS. The slides were blocked in 1 % BSA (Sigma), 0.2 % dry milk powder and 0.1 % Tween 20 in PBS for one hour, followed by the incubation of a 1:100 dilution of the primary antibody in 1 % BSA and 0.1 % Tween 20 in PBS for one hour. Tissue sections were rinsed in PBS and incubated for a further 30 minutes with a 1:400 dilution of the secondary antibody in 1 % BSA and 0.1 % Tween 20 in PBS. The slides were rinsed again in PBS and counterstained with DAPI (1:1000) in PBS for 15 minutes. Sections were

rinsed, mounted with Immu-mount (Shandon) and viewed under the fluorescent microscope.

Immunoenzyme staining: 5 μm tissue sections were incubated in 3% oxygen peroxide for 10 minutes, washed and then pepsin antigen retrieval was performed at 37°C for one hour. Samples were blocked in 10% normal goat serum (Vector Laboratories) and 0.1 % Tween 20 in PBS for one hour, followed by the incubation of a 1:100 dilution of the primary antibody in 1 % normal goat serum and 0.1 % Tween 20 in PBS for one hour. Tissue sections were rinsed in PBS and incubated for a further 30 minutes with a 1:200 dilution of the secondary antibody in 1 % normal goat serum and 0.1 % Tween 20 in PBS. The slides were washed in PBS and incubated for 10 minutes with the Vectastain ABC kit (Vector Laboratories) and followed by a further rinse with PBS. The slides were incubated with a 1:10 dilution of DAB/Metal concentrate (10x) (Pierce) in stable peroxidase substrate buffer (1x) (Pierce) until a brown colour appeared which was followed by a further wash with PBS. The samples were counterstained with Mayer's Haematoxylin for 1 minute, washed, incubated in 1% HCl/70% ethanol for 3 seconds, rinsed in tap water for 5 minutes and then dehydrated and mounted. Unless stated, all incubation and washing steps were performed at room temperature. For immunostaining on paraffin-embedded sections with collagen type II and VI antibodies, pepsin antigen retrieval (Pepsin Solution, Lab Vision Corporation) at 37°C for 10 minutes was performed.

Proteoglycan determination. The proteoglycan contents of the samples were measured as sulphated glycosaminoglycan (GAG) by a direct spectrophotometric microassay with Dimethylmethylene Blue (DMMB) with chondroitin sulphate from bovine trachea (Fluka BioChemica) as a standard. GAG contents were determined in pellets cultivated for 2, 3, 4,

5 and 6 weeks under normal conditions. Two independent experiments were performed and measured in duplicates.

Assessment of cell death. Cell death was determined on paraffin sections of pellets incubated for 4 weeks using the 'In Situ Cell Death Detection Kit, POD' (Roche Applied Science). Briefly, the sections were deparaffinised in xylol and rehydrated through graded ethanol. The sections were then incubated with nuclease-free proteinase K (Roche Applied Science) for 30 min at 25°C. After rinsing in PBS, the sections were incubated with the labeling mix for 1 h at 37°C as recommended by the manufacturer. After rinsing in PBS the sections were counterstained with DAPI in PBS (1:1000) for 10 min at 25°C. Sections treated with DNase I served as positive controls and resulted in > 90 % of dead cells. Two independent experiments were performed and at least 300 cells per pellet were counted to determine the ratio of life vs. dead cells.

6.2. Detection of RAGE at protein and gene level

6.2.1. Introduction and aim

In order to corroborate our immunohistochemistry data, we proceeded to identify RAGE at protein and gene expression level in human tissue as we were unable to detect it in bovine NP cells as shown in Paper 1.

6.2.2. Results

Unfortunately by immunoblotting we were unable to detect RAGE using two commercially available antibodies, rabbit polyclonal antibody for RAGE (Anaspec) and

mouse monoclonal antibody for RAGE (Chemicon). The mouse monoclonal antibody had been used for the immunohistochemistry experiments and had given a positive signal. For immunoblotting, 80µg of protein from a human degenerated intervertebral disc was loaded. Immunoprecipitation was further performed to analyse the presence of RAGE. Both approaches failed and no RAGE could be detected in the human degenerated IVD lysate. Human lung tissue served as a positive control for RAGE (Figure 11).

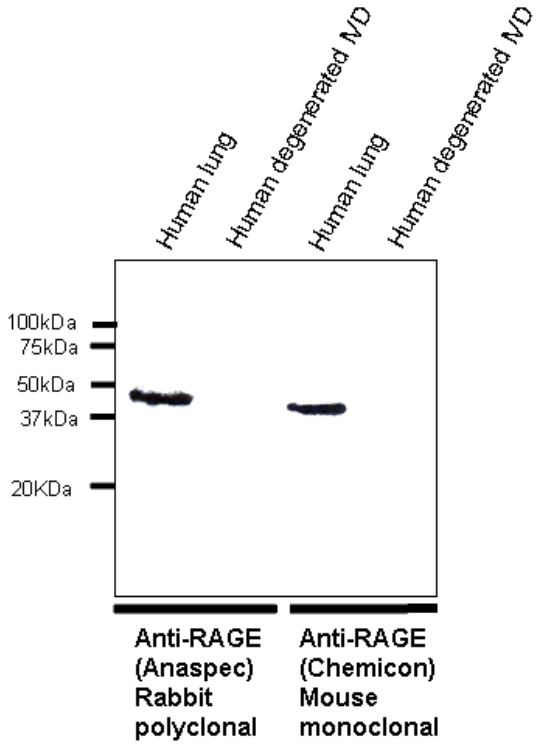


Figure 11. Immunoblotting to detect RAGE in human degenerated IVD and lung tissue. Lysates prepared from human lung tissue served as a positive control. RAGE could not be detected in the human degenerated IVD biopsy. Ponceau red was applied to check for loading.

Conventional RT-PCR was additionally performed on human lung, and biopsies of 6 human degenerated IVDs. The positive control was again positive. However RAGE could

not be detected in neither of the human degenerated IVDs, even after 40 cycles (data not shown).

Taken together these findings indicate that the RAGE protein is unidentifiable under denaturing conditions such as immunoblotting or immunoprecipitation, as well as at mRNA level as shown by the RT-PCR.

6.2.3. Materials and methods

The materials and methods are the same to the submitted manuscripts.

6.3. NGF in the intervertebral disc

6.3.1. Introduction and aim

Recently, an atypical growth factor has been found inside the human intervertebral disc, which is the nerve growth factor (NGF). NGF does not have any known proliferative or anabolic functions on disc cells but has been shown to exert neurotrophic properties in other tissues. Additionally, as described by Abe *et al.* there is a low but constitutive expression of NGF and its receptor TrkA in human NP cells [337]. Moreover, both, IL-1β and TNF-α, up-regulate its gene expression and protein secretion into the media as well as upregulating TrkA expression in NP cells. The exact role of NGF produced by IVD cells in the generation of discogenic pain or on the metabolism of IVD cells is not clearly elucidated. Furthermore, its presence in degenerated intervertebral disc as described in the literature is rather contradictory as NGF has until recently been thought to be produced

exclusively by neurons. In a study by Freemont *et al.*, immunoreactivity to NGF was found exclusively in pain level discs, whilst non-pain level discs or normal discs were entirely negative [338].

Growth factors are being brought to our attention due to their putative contributions to disc degeneration and pain induction. The aim of this experiment was to analyse NGF at protein level in bovine disc cells and to test whether the expression could change in response to a specific stimulation: the accumulation of advanced glycation end-products, low pH (6.7) and oxidative stress due to peroxynitrite.

6.3.2. Results

Immunoblotting analysis has shown the presence of the precursor form of NGF in bovine nucleus pulposus cells. In whole cell extracts, no changes on expression were detected upon stimulation for either 40 minutes, 3 or 6 hours (data not shown). However, cytoplasmic extracts prepared from bovine NP cells stimulated for 40 minutes did show an increase in the NGF precursor form when bovine NP cells were stimulated with either low pH, SIN-1 (*i.e.* stable peroxynitrite donor) and TNF-α (Figure 12).

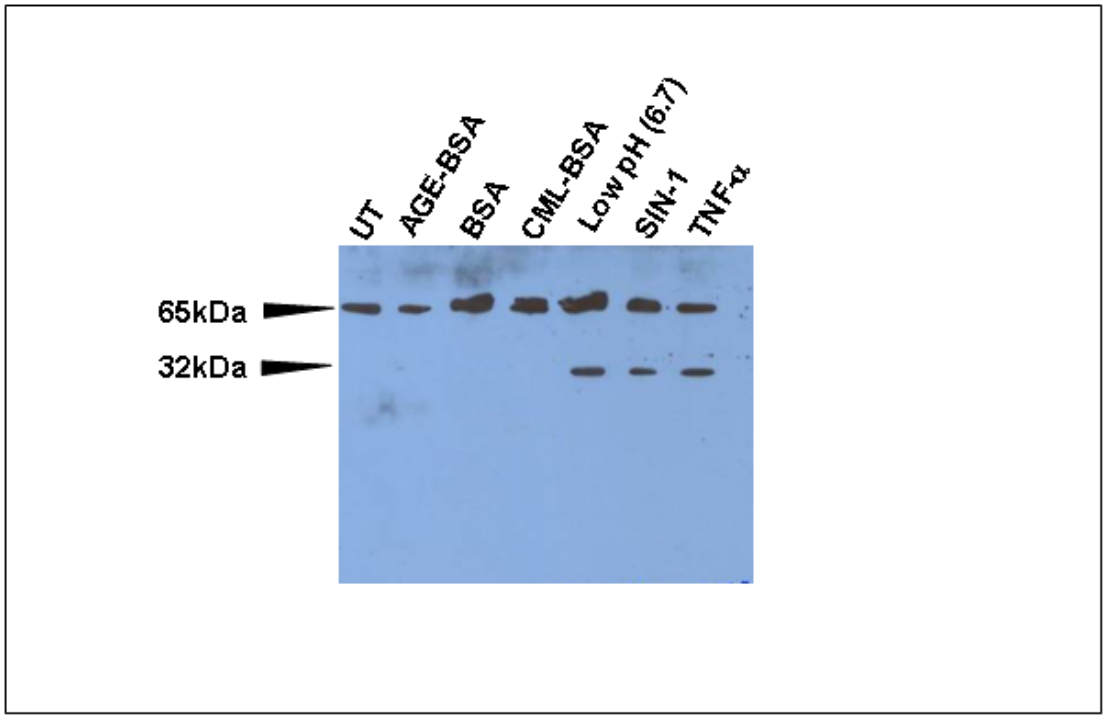


Figure 12. NGF expression in bovine NP cells. Cytoplasmic extracts of bovine NP cells stimulated for 40 minutes under different conditions. A 65kDa band corresponding to p65(RelA) can be detected which served as a control. Typically, p65(RelA) should be cytoplasmic in unstimulated cells, upon stimulation like with TNF-α, p65(RelA) translocates into the nucleus even though a pool of p65(RelA) still remains in the cytoplasm. 40μg of cytoplasmic fraction was loaded (based on Ponceau red staining). The precursor form of NGF can be detected at 32kDa under certain conditions.

6.3.3. Materials and methods

The materials and methods are the same to the submitted manuscripts.

6.4. Influence of hypoxia (0.2% oxygen), low pH (6.7) and SIN-1 on bovine NP cells

6.4.1. Introduction and aim

The intervertebral disc is the largest avascular tissue in the human body. Although some vascularisation of the disc exists during the fetal and infantile stages of life, the tissue

becomes avascular during youth and remains this way thereafter. The blood vessels originating in the vertebral body and originally reaching into the disc, terminate right above the cartilage EP, leaving the disc cells without direct supply [339, 340]. Oxygen and nutrients need to diffuse from the capillaries of the vertebral bodies through the EP and disc matrix into the nucleus of the disc, while waste diffuses in a same manner, outwards. Measurements of oxygen and lactic acid concentrations in IVDs of patients suffering from low back-pain or scoliosis showed that lactic acid accumulates with increasing distance from the boundary of the disc [341]. Whilst oxygen concentrations were very low in the nucleus and increased towards the disc surface, the lactic acid concentration showed a reverse profile. Lactic acid is not only the major waste of disc cells, but it is an acid and thus able to lower the pH inside the IVD. *In vitro* experiments have shown that low oxygen concentrations and an acidic pH significantly affect the activity of disc cells. Matrix synthesis rates were observed to decrease significantly and cell viability was also adversely affected [342].

These preliminary experiments aimed to examine the pro-inflammatory cytokine profile in bovine NP cells under a hypoxic environment (0.2% oxygen) using the same set up as in Paper 1 (section 5.1.) (Figure 13), or in an acidic environment, *i.e.* media with a pH = 6.7, or oxidative stress caused by peroxynitrite using the stable peroxynitrite-donor SIN-1 (1mM) (Figure 14).

6.4.2. Results

These preliminary experiments indicated that hypoxia induces changes in the expression profile of IL-1β only as shown in Figure 13 especially at 6 hours with CML-

BSA. There were no changes for either IL-6 (Figure 13) or TNF-α (data not shown) expression. Obviously, a dramatic decrease oxygen tension down to 0.2% oxygen greatly affects IL-1β expression levels in the presence of the advanced glycation end products, CML, which has huge consequences in ECM remodelling processes. With regard to the oxygen tension, the centre of the NP region of the IVD has relatively low levels of oxygen (as shown in Fig. 2); nevertheless a further decrease due to possible EP calcification might be rather detrimental in trying to endure ECM homeostasis.

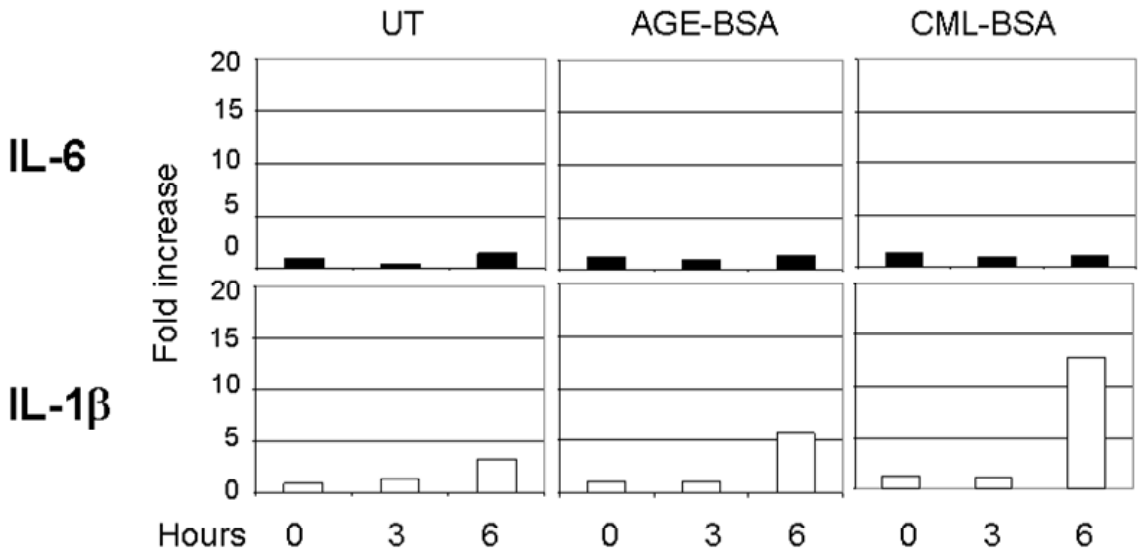


Figure 13. Pro-inflammatory cytokine profile under hypoxia of bovine nucleus pulposus cells stimulated with AGE/CML-BSA. All experiments were performed under hypoxic conditions at 0.2% oxygen. The cytokine profile for IL-6 and IL-1β was analysed using quantitative real-time RT-PCR. NP cells were stimulated with either AGE/CML-BSA. On the *y* and *x* axis the fold increase and the time point in hours is represented, respectively. Expression levels were expressed in arbitrary units, setting the untreated value as 1. The values were normalised relative to the reference gene S18 protein mRNA to compensate for differences in cDNA loading. This figure represents a mean of at two independent biological samples.

An environmental acidic pH or the damage caused by the oxidising agent, peroxynitrite, induces changes only in IL-1β gene expression. Instead, IL-6 (Figure 14) and TNF-α (data not shown) remained again unchanged. These results indicate that both stimuli

are inducing the pro-inflammatory cytokine IL-1β mainly implying that IL-1β may be key in the degenerative processes occurring in the IVD.

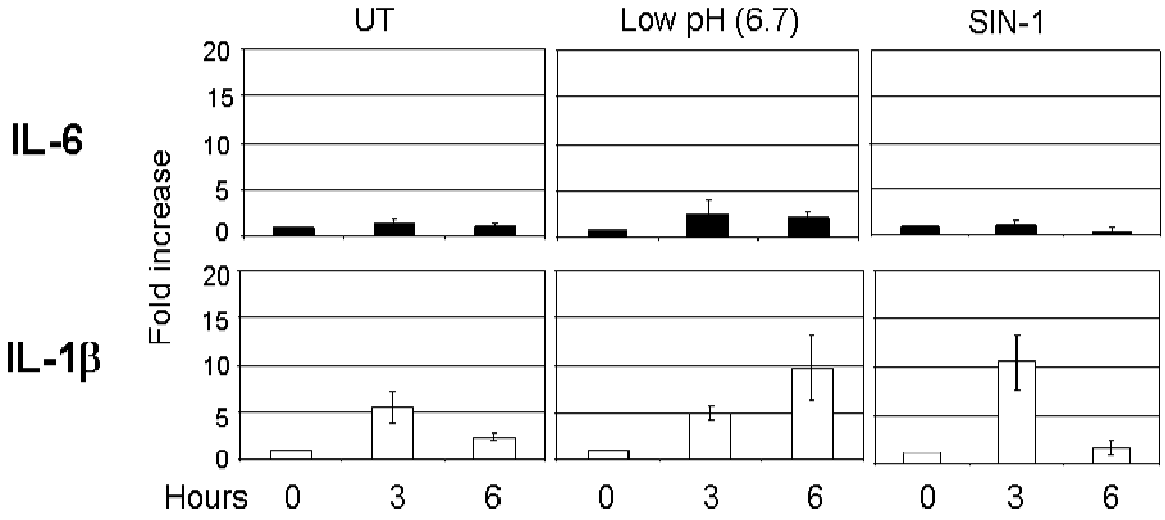


Figure 14. Pro-inflammatory cytokine profile of bovine nucleus pulposus cells stimulated with a low environmental pH (6.7) and SIN-1. All experiments were performed under normoxic culturing conditions. The cytokine profile for IL-6 and IL-1β was analysed using quantitative real-time RT-PCR. NP cells were stimulated with either a media with pH 6.7 or by 1mM of SIN-1. On the *y* and *x* axis the fold increase and the time point in hours is represented, respectively. Expression levels were expressed in arbitrary units, setting the untreated value as 1. The values were normalised relative to the reference gene S18 protein mRNA to compensate for differences in cDNA loading. This figure represents a mean of at three independent biological samples.

6.4.3. Materials and methods

The materials and methods are the same to the submitted manuscripts.

6.5. Effects of short and long-term peroxynitrite stimulation in nuclear shuttling of NFκB/p65

6.5.1. Introduction and aim

Preliminary immunofluorescence experiments were performed to determine the prolonged effects on nuclear translocation of NF-κB/p65 by the peroxynitrite donor SIN-1 (1mM) on human NP cells. TNF-α (10ng/ml) was chosen as a positive control for the transient canonical NF-κB pathway.

6.5.2. Results

At 40 minutes immunofluorescence microscopy showed nuclear translocation of p65(RelA) upon TNF-α (10ng/ml) stimulation whereas only a few NP cells had a nuclear staining for p65(RelA) when treated with SIN-1. In contrast, at 14 hours, the pattern changed, as in TNF-α treated cells, staining was mainly cytoplasmic, while the majority of SIN-1 stimulated cells had a nuclear as well as cytoplasmic staining for p65(RelA). The staining for both time points in unstimulated cells was mostly cytoplasmic. Even though the morphology of the cells changed a bit due to the starvation of these cells during the time of stimulation, *i.e.* no serum was added to the media, cell viability was >90% as detected by Calcein-AM and Ethidium Homodimer staining.

These results indicate that peroxynitrite is involved in a prolonged nuclear translocation of p65(RelA) which could potentially be activated and be involved in chronicity or prolonged inflammatory processes.

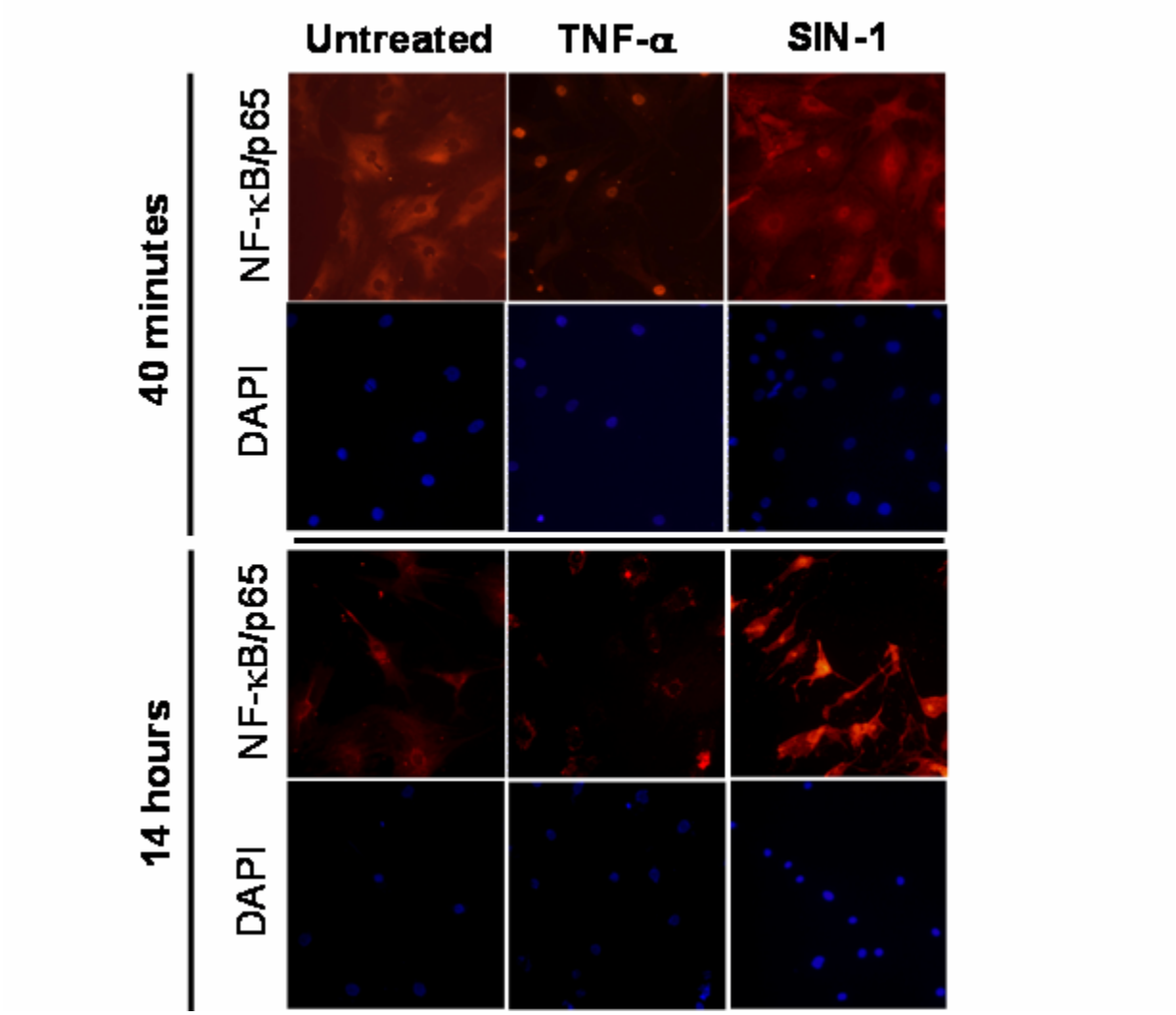


Figure 14. Detection of p65(RelA) in human NP cells by immunofluorescence. Human NP cells were stimulated for 40 minutes or 14 hours with either TNF-α (10ng/ml) or SIN-1 (1mM). Red staining corresponds to p65(RelA), the nucleus of the cells were counterstained with DAPI (blue). The experiment was performed in cells from degenerated NP cells from two different donors (n=2).

6.5.3. Materials and methods

The materials and methods are the same to the submitted manuscripts.

6.6. Human telomerase reverse transcriptase (hTERT) in NP cells

6.6.1. Introduction and aim

In vitro culture systems are urgently required to permit the study of the molecular processes leading to IVD degeneration and ultimately, pain. Unfortunately, the slow rate of growth, the halt in growing cultures after a certain passage and the differentiation processes of human NP cells associated to monolayer culture, greatly hamper experiment. In fact, human NP cells have been reported to arrive to a senescence state at approximately passage 20 [343]. 3-D systems permit the circumvention of differentiation problems; however, these systems involve a limited number of cells and the 'noise' produced by the introduction of foreign matter.

A recent tool to immortalise human NP cells is the overexpression of the human telomerase catalytic subunit (hTERT) [344, 345]. Immortalisation of cells with hTERT has an advantage over viral proteins. The viral immortalisation mediators display oncogenic properties, such as inactivation of the major tumour suppressor proteins [346]; hTERT solely elongates chromosomal ends, which prevents genomic catastrophe or crisis associated with cell death [347]. It is well accepted that early passage hTERT-immortalised cells, for instance in propstate stromall cells 90 passages, faithfully represent the physiologic properties of normal cells *in vivo* [348].

Thus, this strategy was chosen to set a ready pool of human NP cells which will maintain their phenotype when cultured in monolayer and which might still be grown after passage 20. This would also help the reproducibility of our experiments. Otherwise the biopsies

obtained from the clinic correspond to a wide spectrum of aged-patients with a wide variety of pathologies.

6.6.2. Lentivirus containing hTERT

For the generation of viruses a total of $4x10^6$ HEK 293T cells were seeded in 10cm dishes 24 hours prior to transfection. Viral stocks were prepared by transient transfection of HEK 293T cells with 3.5μg of the envelope plasmid, 6.5 μg of packaging plasmid, and 10μg of transfer vector using Calcium phosphate precipitation method (Plasmids used: Protein G, NGVL *gag pol*, and the pOS containing hTERT vector, respectively). The medium of HEK 293T cultures was replaced 5 hours post-transfection and conditioned medium was collected after another 24 hours, filtered through a 0.45μm cellulose acetate filters. Freshly harvested conditioned medium was used to infect 1.5x105 NP cells in a six-well plate in the presence of polybrene (4μg/ml) (Sigma, Switzerland).

This protocol was adopted from [349]. Cells were passaged twice and analysed by immunofluorescence to check for GFP incorporation, followed by FACS sorting. Positive and negative GFP containing cells were further plated to expand. Retroviral pOS vectors were kindly provided by Prof Zimmermann (Freiburg, Germany).

The source of NP cells came from two donors: a sequestered-herniated IVD from a 40-year-old male, and a young IVD from a 14-year-old boy with spinal fracture which would otherwise be considered healthy.

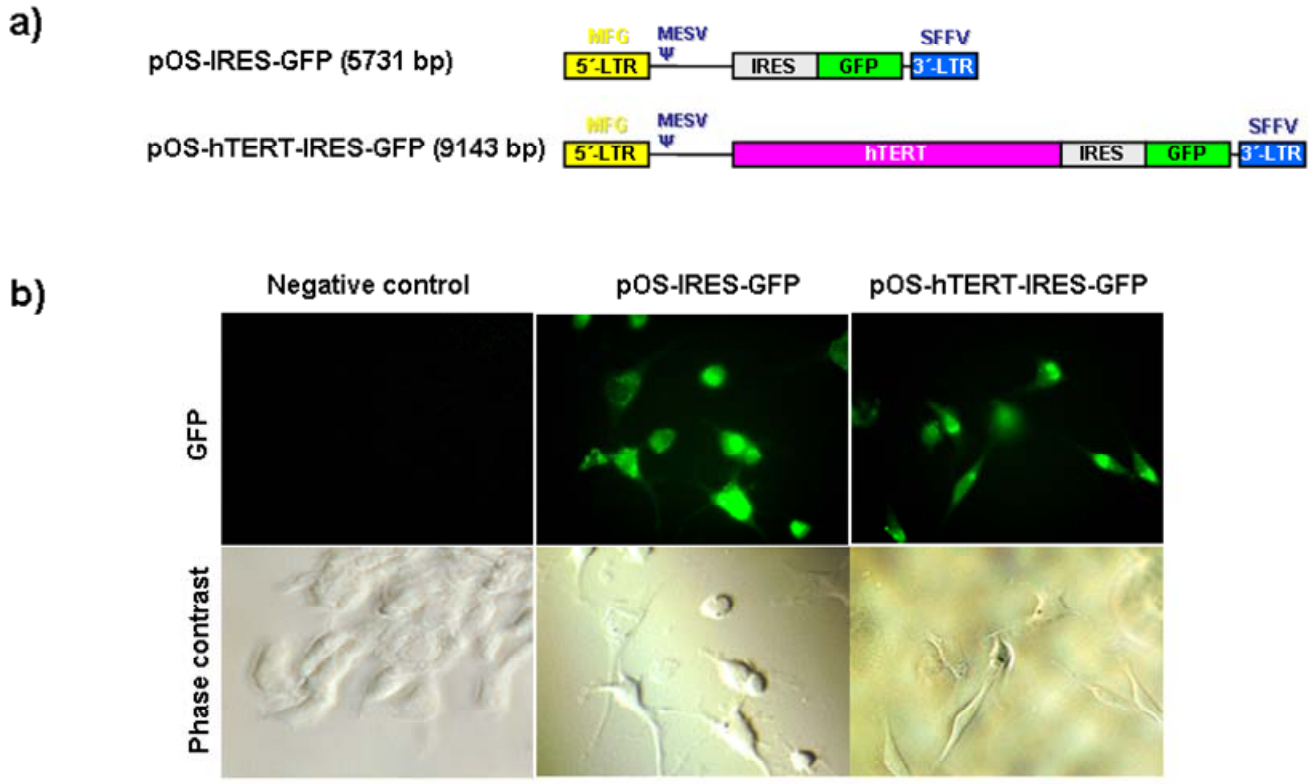


Figure 16. Retroviral pOS vectors containing hTERT. a) Schematic diagram of the pOS vectors. b) Immunofluorescence due to GFP of the 40-year-old IVD cells representing the success upon transfection with the lentivirus containing the pOS vectors. The first column corresponds to the untransfected cells. Same pictures were obtained for NP cells of the 14-year-old donor.

6.6.3. Results

The cells had a transfection rate which varied from 5 to 10%. Transfected cells with the retroviral pOS vectors immunofluoresced green for GFP (Figure 16). This is an ongoing project. NP cells were further expanded until passage 5 and handed over to Dr. Wuertz. Phenotypic characterisation and telomere length measurement will be assessed in the near future.

DISCUSSION

7. Summary of results

Experimental evidence presented in this thesis identified putative mechanisms involved in the degenerative processes possibly occurring in the ageing disc by the action of oxidative stress. This involves post-translational modifications: advanced glycation end products (AGEs) and tyrosine nitration mediated by peroxynitrite.

The *first paper* provides evidence for the role of AGEs in the ECM remodelling/disruption processes as shown in the NF-κB-dependent metalloproteinase MMP-13. Although the examined AGE receptors were not affected by its ligands AGE/CML in the bovine model; on AGE stimulation NF-κB/p65 nuclear translocation occurred, implying the putative activation of an NF-κB-dependent pathway in the IVD.

In the *second paper*, tyrosine nitration was observed in human NP cells from degenerated IVDs. Tyrosine nitration is a marker for peroxynitrite, which is a highly reactive agent formed *in vivo* from the interaction of superoxide and NO. Both, peroxynitrite and SIN-1 (a stable peroxynitrite donor) induced nitrosylation in NP cells as well as the activation of a prolonged NF-κB-dependent signalling pathway, resulting in the upregulation pro-inflammatory cytokines IL-1β, IL-6 and IL-8. The prolonged nuclear shuttling of NF-κB by peroxynitrite in NP cells might be indicative of its putative involvement in a chronic inflammation.

The unpublished results provide a characterisation of the chondron-pellet system indicating its limitations for experimental use in cell number and time viability course. Additionally, human NP cells were infected with a lentivirus containing hTERT, in order to

develop a human NP pool of cells to use in future experiments which do not readily differentiate. hTERT should elongate the telomeric ends allowing a higher passage number; nevertheless, this is part of an ongoing project which will require phenotypic characterisation of these NP cells in the future.

Taken together, the results presented in this thesis provide further evidence that NF-κB activation is central and can potentially influence ageing/degeneration processes in the IVD through AGE accumulation or peroxynitrite action.

8. A chondron-based pellet culture system of bovine NP cells.

Initially, we attempted to establish a 3-D system which could potentially be used for our intended experiments because of the shortcomings of 2-D systems as discussed in section 1.5.1. Nevertheless, after encountering significant limitations with our best choice of 3-D culture system, we decided to change our approach by culturing IVD cell in monolayer culture limiting the passage number of the cells to 4. The corresponding phenotypic characterisation was performed to ensure that the phenotypic markers were still expressed.

8.1. Use and limitations

Since it has been shown that disc cells require a three-dimensional cultivation system in order to maintain their phenotype, a modification of the carrier-free pellet culture system in this study was applied. Based on the original pellet culture system presented by Yung Lee *et al.* [146], we assumed that the inclusion of the native PCM of the IVD cell in the pellet formation process may further ensure the conservation of the native cell phenotype. From studies on articular cartilage, the concept of the 'chondron', i.e. the chondrocytic cell surrounded by the PCM, has been established [146, 334]. Therefore, we applied a pellet culture system that combines the advantages of the original pellet system with the chondron concept. In our approach, we made use of the easy availability of bovine coccygeal nucleus pulposus cells that have been shown to display a cellular phenotype similar to their human counterparts [148, 332]. Histology demonstrated that disc cells cultured in this system shared properties with the native disc cell phenotype, *i.e*. low collagen type I expression, collagen type II expression and presence of proteoglycans.

Dedifferentiation was characterised by alterations of the morphological appearance of the cells and the presence of the fibrillar collagens in the outer rim and after prolonged incubation. Taken together, the histology data suggest the chondron-based pellet system might be a suitable model to culture disc cells *in vitro*, even though the number of cells that can be used per pellet is rather limited, hampering the range of experiments that can be performed.

To accelerate CML formation in the pellet culture, glucose in the standard cultivation medium was replaced by ribose, a more reactive carbohydrate that has been shown to react spontaneously with amino groups on proteins to form AGEs [350]. The presence of ribose in the medium led to a pronounced accumulation of CML in the pellet matrix. Accordingly, the highest levels of RAGE were also observed in pellets cultured in the presence of ribose. Under these conditions we found that the majority of the cells in the pellets were dead at the end of the experiment after 4 weeks of incubation (> 90 %). Thus, it can be postulated that ribose exerts adverse effects on NP cells. In pellets incubated in standard medium, slightly reduced levels of CML and RAGE were found compared to the ribose treated cultures. Considering the significantly lower reactivity of glucose compared to ribose, a substantially lower accumulation of CML in the matrix is to be expected. It is conceivable that the high glucose concentration present in the normal culture medium induces intracellular reactive oxygen species (ROS) as seen with diabetic complications [351]. ROS might not only be responsible for the substantial CML and RAGE levels but might also lead to apoptosis of the cells which explains the high percentage of dead cells (approx. 50 %). In the absence of glucose in the culture medium, CML accumulation and RAGE expression decreased dramatically and were hardly detectable. Culture under hypoxic or acidic conditions in the

presence of glucose resulted in CML accumulation and RAGE expression that was more pronounced than under glucose-free cultivation conditions but reduced in comparison with the standard conditions. The decreased CML and RAGE levels under hypoxic or acidic conditions might be interpreted as a protective effect of low pH and pO_2 hindering CML accumulation in the presence of high glucose levels. However, culture under hypoxic, acidic and no-glucose conditions resulted in approximately 50 % of dead cells in the pellets. The poor cell survival under all applied cultivation conditions suggests that the chosen cultivation period is too long. Further experiments with shorter incubation periods should allow determination of CML accumulation and RAGE expression at a time point when the majority of the cells are still alive.

Preliminary experiments have indicated a 90% viability of cells under most conditions at 2 weeks incubation, implying that a shorter incubation may be desirable for the use of chondron-pellet culture system. Although the cell survival in this setup needs to be improved, these data suggest that CML accumulation and RAGE expression can be modulated by the applied culture conditions. It is important to keep in mind that cell viability has only been determined at the end of the experiment after four weeks of incubation. Therefore, we do not yet have any information about the time course of cell death. Substantial differences in CML accumulation and RAGE expression with comparable levels of cell death might suggest that the environmental condition caused CML accumulation and RAGE expression earlier during the experiment, when the cell were still alive. Experiments with shorter incubation periods should further clarify the time course of CML accumulation and RAGE expression versus cell death.

8.2. Perspectives

Nevertheless, the above data on RAGE immunohistochemistry has to be carefully taken into account as we could not detect RAGE by immunoblotting or immunoprecipitation. It could not even be detected by RT-PCR. Both antibodies used in these experiments can detect non-denatured and denatured proteins. Various possible explanations include:, i) the mouse monoclonal antibody for RAGE from Chemicon might have been isolated with some other protein, as a high molecular weight is observed in bovine lung (approx. 150kDa), or ii) that RAGE itself is glycosylated *i.e.* incrementing its size, iii) that RAGE itself dimmerises or forms some kind of non-covalent complexes, or finally iv) in the case of the bovine RAGE there is a high molecular weight splice variant.

9. Role of AGEs in disc matrix homeostasis and remodelling processes.

9.1. ECM remodelling

Advanced glycation end products (AGEs) result from non-enzymatic glycation of the amino groups of proteins with reducing sugars such as glucose. During normal ageing, but also at a higher rate during complications involving inappropriate sugar reabsorption such as diabetes, AGEs accumulate on long-lasting proteins such as collagens and elastins. The formation of AGEs on collagen favours its cross-linking, resulting in a decreased degradability by, *e.g.*, matrix metalloproteinases (MMPs), and impairing their regeneration. Collagen metabolism is a complex process requiring balanced synthesis and degradation by ECM-remodelling enzymes and the action of cytokines. In our study we analysed the influence of AGEs in NP cells by examining receptor profile, pro-inflammatory cytokine gene expression, nuclear translocation of NF-κB/p65 and their influence on a key degradative enzyme, MMP-13. Receptor and cytokine expression did not vary upon stimulation with AGEs. Nuclear translocation NF-κB/p65 was indeed observed and MMP-13 expression was largely enhanced. Thus, our results indicated that AGEs may induce an NF-κB-dependent pathway and target genes comprising ECM-remodelling enzymes. Even though, it should be noted that a direct link between NF-κB and MMP-13 was not analysed, MMP-13 has been reported to be a NF-κB-dependent gene and was even reported to be upregulated in degenerated IVD [352]..

These findings suggest that due to AGE accumulation in long-lived-ECM proteins, possibly causing tissue brittleness and stiffness to the tissue, the cells in the IVD might be trying to counteract this by producing a surplus of MMP-13. This would then lead to a shift towards catabolic processes. Nevertheless, this might not sufficient or effective in restoring the natural properties and function of the ECM of the IVD. A reverse effect might even occur due to this surplus of MMP-13 being produced, as it might induce the degradation of other healthy collagens.

9.2. Perspectives

AGEs accumulate in aging/degenerative tissues including the IVD. This accumulation is enhanced under certain pathologies involving sugar excesses as in diabetes mellitus.

The effects exerted by these post-translational modifications on ECM-long-lasting proteins are obvious as it renders the tissue stiff and brittle, leading to a loss of function, *i.e.* unable to withstand and distribute forces. Undoubtedly, the accumulation of AGEs is a well-established and important feature of age-related IVD.

With regard to a receptor-mediated pathway, we were able to identify three receptors which have been reported to form the so-called AGE-receptor complex. Although their expression did not vary upon stimulation, further experiments analysing the interaction between AGEs and the AGE-receptor complex remain to be performed. Contrary to previous publications [353, 354], RAGE *i.e.* the best characterised AGE receptor, which has a tissue specific distribution, could not be detected under the conditions tested..Additionally, Schmitt et al. identified up to 10 proteins which could bind to AGEs [355]. For instance, it has been

reported that only a partial reduction of AGE-stimulated NO and chemokine release can be obtained by blocking RAGE [356]. Thus, the total binding of AGEs to cell surface proteins, and not only the binding to RAGE, might be important for mediating AGE-effects on cells. So, if ligand binding interactions with the AGE-receptor complex fail, it is possible that unidentified AGE-receptors are signalling in NP cells.

A useful *in vivo* model to study the overall effects of AGEs on IVD cells might be the next step to proceed, as *in vitro* experiments are rather limiting only allowing the analysis of certain parameters. *In vivo* models that would enable the analysis of more profound effects involving pain induction and/or sensitation of the dorsal root ganglion may involve the sand rat or other diabetic animal models as discussed in section 1.2.3. Of course these models are not the best, since many metabolic disorders accompany diabetes mellitus, but they imply an enhanced accumulation of AGEs in the IVD at a younger age. Additionally, the sand rat has already reported in the literature to have very similar degenerative changes to humans [357].

AGEs seem to be an important feature in age-related changes. However our results indicate that AGEs may not be involved in the pro-inflammatory pathways in the IVD. Instead AGEs may be disturbing the ECM properties of the IVD leading to a loss of function.

10. Peroxynitrite induces cytokine gene expression in human IVD cells

10.1. Peroxynitrite and the activation of an alternative NF-κB signalling pathway.

The constitutive nuclear translocation due to peroxynitrite or SIN-1 treatment could be observed by immunofluorescence up to 14 hours. This prolonged mode of action is in accordance to recent data that demonstrate that prolonged exposure to peroxynitrite and/or other reactive nitrogen species continuously activates NF-κB through tyrosine nitration of IκB, leading to inhibited degradation by the ubiquitin-proteasome pathway [302]. The intracellular formation of peroxynitrite from extracellular NO was shown to be crucial for the activation of NF-κB by NO [304]. Recently, NF-κB has been linked to the activation of different intracellular and extracellular proteolytic systems under conditions of nitrosative stress [290]. Hence, this continuous and long-term activation of NF-κB may exacerbate the inflammatory responses mediated by this transcription factor. This was shown for various cell types including muscle myoblasts and epithelial cells [302, 321].

By site-directed mutagenesis, tyrosine 181 was identified as the main nitrosylated residue in IκBα. IκBα tyrosine 181 nitration leads to dissociation of IκBα from NF-κB [321]. This mechanism does not require IκBα kinase (IKK) dependent phosphorylation or proteolytic degradation. The importance of tyrosine 181 is its ability to stabilise the IκBα-NF-κB complex through non-covalent interactions with the p50 subunit as shown by the crystal structure[321]. Thus, tyrosine nitration becomes an important post-translational modification involved, in this case, in an aberrant NF-κB activation.

10.2. Target genes of peroxynitrite.

The genes affected by peroxynitrite consist of a wide spectrum of target genes which include matrix remodelling proteins (MMPs and TIMPs) and pro-inflammatory cytokines among others. Our target genes of interest in these particular experiments were pro-inflammatory cytokines as they have long been implicated in disc degeneration or pain.

Oscillatory wave curves were detected for three NF-κB-dependent cytokines such as IL-1β, IL-6 and IL-8, indicating that even though NF-κB might be a shared transcription factor for these genes, the recruitment onto the promoter, its accessibility and the involvement of various co-regulators might be rather different, leading to time differences. NF-κB activity itself was not directly analysed. Nevertheless, nuclear shuttling of p65(RelA) was analysed as it might be an indicative of an NF-κB-dependent pathway, since in an inactive state, p65(RelA) is mostly retained cytoplasmic.

The analysis of matrix remodelling proteins might be another interesting mechanism induced by peroxynitrite as this compound formed *in vivo* might exert detrimental effects on tissue homeostasis. Thus, peroxynitrite could potentate age and enhance degenerative processes in the IVD.

10.3. Perspectives

Peroxynitrite and other RNS and ROS arise as a very reactive group of components that can induce severe detrimental effects in a biological system. Due to their low molecular weight they can readily cross cell membranes without limiting the damage that they can exert, since not only exogenous but especially endogenous components can be affected. The action of peroxynitrite was implicated in many cellular processes ranging from DNA

damaging to the constitutive activation of transcription factors as in the case NF-κB. The use of antioxidants or anti-nitrosylating might pose a new therapeutic approach the treatment of degenerative or inflammatory diseases.

The maintenance of an oxidative redox equilibrium might be indeed crucial. Oxidative stress may not only arise from an excess of reactive oxygen/nitrogen species arising from cellular metabolism, but also due to the hampering of appropriate detoxifying agents. Genetic epidemiological studies indicated that there are polymorphisms in the gene encoding vitamin D receptor which correlated with IVD degeneration [58, 59]. So far, little research, has tackled the issue on detoxifying cellular mechanisms in the IVD. Mutations might not be the only factor influencing the anti-oxidant enzymes and receptors. The shifting towards an oxidative stress may also arise from environmental factors such as a dysfunction in nutrition and diffusion of metabolites. Apart from the diffusion of glucose, an impaired nutrition affects many other small molecules such as vitamins e.g. anti-oxidants, minerals, and salts among others; all of which are required for regular cell function. In addition, an acidic pH might induce a dysfunction in the cellular metabolism leading to an increase in oxidative metabolites. Furthermore, a drop in anti-oxidants and a malfunction of their receptors could even lead to cellular senescence. In summary, an insight in the anti-oxidant mechanisms of a cell might be determinant in dealing with IVD degeneration; *i.e.* being decesive when the reversal of degenerative alterations and subsequent pain come into focus for therapeutic purposes.

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ACKNOWLEDGEMENTS

I would like to thanks my supervisor, Norbert Boos, for support throughout my thesis and ensuring a place to perform the remaining final years of my thesis. Special thanks to Michael Hottiger for taking me as a part of his group, helping with constructive criticisms and ideas of this project and agreeing to be my project supervisor and examiner.
I would also like to thank my other supervisor, Roland Wenger for letting me use his hypoxic box and the help of his lab members as well as for his helpful advice and agreeing to be my examiner. I thank Mauro Alini for his good advice and agreeing to correct my thesis as an external member of the university.
I thank all the members of the IVBMB, for all the technical support given in the two years I have spent there, specially to Sarah for her support and showing me where everything is, Karin for all her support and Sandrine for her useful tips, Taras for his help with the retrovirus experiments, Michi ad Ralph for all their IT help and Süheda, Matthias, Radina, Moni and Simon for their inputs during the group meetings. I also would like to thank Karin Wuertz for her help and translating my Zusammenfassung . I thank as well members of the Forschung OK, in the Uniklinik Balgrist for all their help during my first years there. Guenther for his help and corrections of the first manuscript, Adam and Fabien for all their moral support and technical help, Lars, Kerstin, Thomas and Knut for knowing generally where everything is and how it works.
My friend have been a huge support throughout these years. Clara and Lluis have been fab, Jacob and his crazy ideas, Bea for our mutual support, my climbing partner and dearest friend, Marco, for his encouragement. Isa, Moreno and Alex-B for just been there for me. For Maria and Ciccio for they're smile and joy.
My family has given me a constant encouragement and have helped me to go the right way, taught me values in life and love. Blanca's patient phone calls throughout the globe, emotional support and her many visits livened this place up. Fabio has given an unconditional support at all times, lightening my life, and making me smile in tough times.